Defective regulatory T cell function in type 1 diabetes a trait under genetic control?

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DEFECTIVE REGULATORY T CELL FUNCTION IN TYPE 1 DIABETES: A TRAIT UNDER GENETIC CONTROL?

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Abstract

Type 1 diabetes (T1D) is an autoimmune disease, resulting from the specific destruction of the insulin-producing beta-cells in the islets of the pancreas. Islet-specific autoreactive T cells are instrumental in this process and although these cells are present in individuals with and without T1D, their exhibition of a memory phenotype in diabetic individuals indicates they may have been previously activated in these patients. This suggests a breakdown in peripheral tolerance, implying regulatory T cells (Tregs) may be involved. Indeed, CD4+ CD25hi FOXP3+ Tregs do not differ in frequency in T1D, but their function is impeded. This defect is present in both recent-onset type-1 diabetics (ROT1D) and long-standing type 1 diabetics (LST1D) suggesting it is a stable phenotype, possibly under genetic control. Also, it is known that the production and signalling of interleukin-2 (IL-2); a cytokine essential for the maintenance of Tregs, is defective in T1D. The aim of this thesis was to ascertain whether defective Treg function in T1D is genetically determined or a consequence of the disease.

The first section of this thesis examined the effect of a T1D-associated *IL-2RA* single nucleotide polymorphism (SNP) on Treg function, by means of a genotype-immunophenotype study. Non-diabetic donors homozygous for the susceptible allele at this SNP exhibited diminished Treg fitness and suppressive action, suggesting that defective Treg function is a contributing factor in T1D. The latter section utilised T1D-discordant monozygotic twins

and healthy controls to examine Treg function and the IL-2-dependent generation of regulatory type 1 (Tr1) cells. Due to the low number of twins obtained nothing conclusive can be drawn from the study on Treg function. However, the results from the Tr1 generation assay suggest the possible existence of an IL-2 signalling defect in non-diabetic twins.

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Abbreviations

ACs	Accessory cells
ADP	Adenosine diphosphate
AF488	Alexa fluor 488
AF647	Alexa fluor 647
AF700	Alexa fluor 700
AIDS	Acquired immunodeficiency syndrome
AIRE	Autoimmune regulator
AMP	Adenosine monophosphate
APC	Allophycocyanin/ Antigen presenting cells
APC-Cy7	Allophycocyanin with cyanine dye 7
APS II	Autoimmune polyendocrine syndrome type 2
ATP	Adenosine triphosphate
aTreg(s)	Activated Treg(s)
BB rat	Biobreeding Wistar rat
BSA	Bovine serum albumin
B6 mouse	C57BL/6 mouse
C-peptide	Insulin connecting-peptide
cAMP	Cyclic adenosine monophosphate
CCL1	Chemokine (C-C motif) ligand-1
CCL2	Chemokine (C-C motif) ligand-2
CpG	Cytosine phosphate guanine
cpm	Counts per minute

CREM	cAMP responsive element modulator
Csk kinase	C-src tyrosine kinase
CTL	Cytotoxic T-Lymphocyte
CTLA-4	Cytotoxic T-Lymphocyte Antigen 4
CXCL10	Chemokine (C-X-C motif) ligand-10
DCs	Dendritic cells
DMSO	Dimethyl sulfoxide
dPBS	Dulbecco's phosphate buffered saline
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
Erk	Extracellular signal-related kinase
FACS	Fluorescence activated cell sorting
FCS	Foetal calf Serum
FDR	First-degree relatives
FITC	Fluorescein isothiocyanate
FoxP3/FOXP3	Forkhead box Protein 3
FSC	Forward side scatter
GAD65	Glutamic acid decarboxylase 65
GADA	GAD65 antibody
GITR	Glucocorticoid-induced tumour necrosis factor receptor-related protein
GvHD	Graft versus host disease
GWAS	Genome-wide association study
hAB	Human AB serum

HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
IA-2	Insulinoma-associated protein 2
ICER	Inducible cAMP early repressor
Idd3	Insulin-dependent diabetes susceptibility locus 3
IDO	Indoleamine 2,3-dioxygenase
IFIH1	Interferon-induced helicase
IFNγ	Interferon gamma
IL-1β,- 2, -7, -10, -12, -15, -17	Interleukin-1 beta ,-2, -7, -10, -12, -15,-17
IPEX	Immune dysregulation, polyendocrinopathy, enteropathy, X-linked
ISIS	IL-10-secreting islet-specific
iTreg(s)	Induced Treg(s)
IU	International units
JA	Juvenile arthritis
Lck kinase	Lymphocyte-specific protein tyrosine kinase
LNs	Lymph nodes
LPS	Lipopolysaccharide
LST1D	Long-standing Type-1-Diabetes/diabetic

LYP	Lymphoid-specific phosphatase
MACS	Magnetic activated cell sorting
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
ml	Millilitre
mm	Millimeter
mM	Millmoles
MS	Multiple sclerosis
mTreg (s)	Memory Treg(s)
NF-κB	Nuclear factor-kappa B
NHS	National Health Service
NK	Natural killer
nm	Nanometres
NOD mouse	Non-obese diabetic mouse
nTreg(s)	Naturally-occurring Treg(s)
PB	Pacific blue
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PE	Phycoerythrin
PerCP-Cy5.5	Peridinin chlorophyll protein with cyanine dye 5.5
pg	Picogram
PHA	Phytohaemagglutinin
PI	Pro-insulin

PPI	Pre-pro-insulin
PLN(s)	Pancreatic lymph nodes
P/S/F	Penicillin/Streptomycin/Fungizone
pSTAT5a	Phosphorylated signal transducer and activator of transcription 5a
PTPN2	Protein tyrosine phosphatase, non-receptor type 2
PTPN22	Protein tyrosine phosphatase, non-receptor type 22
QDot	Quantum dot
RA	Rheumatoid arthritis
ROS	Reactive oxygen species
ROT1D	Recent-onset Type-1-Diabetes/diabetic
RPMI	Roswell Park Memorial Institute medium
RRMS	Relapsing-remitting multiple sclerosis
rTreg(s)	Resting Treg(s)
sCTLA-4	Soluble cytotoxic T-Lymphocyte Antigen 4
S.D.	Standard deviation
SLE	Systemic lupus erythematosus
SSC	Side scatter
SNP(s)	Single-nucleotide polymorphism(s)
SPAK	Ste 20-related proline alanine-rich kinase
STAT5a	Signal transducer and activator of transcription 5a

T1D	Type 1 diabetes
Tconv	Conventional T cell(s)
TCR	T cell receptor
TGFβ	Transforming growth factor beta
T_H1	T-helper cell (type 1)
T_H2	T-helper cell (type 2)
T_H3	T-helper cell (type 3)
T_H17	T-helper cell (type 17)
T_H22	T-helper cell (type 22)
TLR	Toll-like receptor
TMB	Tetramethylbenzidine
TNFα	Tumour necrosis factor-alpha
Tr1 cell(s)	Type 1 regulatory cell(s)
Treg(s)	Regulatory T cell(s)
TSDR	Treg-specific demethylated region
VNTR	Variable number of tandem repeats
X-SCID	X-linked severe combined immunodeficiency syndrome
ZAP-70	Zeta-chain-associated protein kinase 70
μg	Microgram
μl	Microlitre

3[H]	Tritium
7-AAD	7-Aminoactinomycin D

Chapter One: General introduction

1.1 Immune regulation

The immune system has evolved over time to protect the host from pathogenic organisms. From the general protection provided by innate immunity in both invertebrates and vertebrates to the specific protection afforded by adaptive immunity, present in vertebrates alone (Cooper and Herrin, 2010). Importantly the immune system is able to distinguish between self and non-self (*i.e.* host tissues and non-host tissues) (Medzhitov and Janeway, 2000) destroying the latter, as demonstrated by transplant rejection (Lombardi and Lechler, 1991), and is capable of doing so rapidly. The immune system is essential for health as illustrated by individuals with compromised immune systems, such as those with X-linked severe combined immunodeficiency (X-SCID) (Kumaki *et al*., 2000) or acquired immunodeficiency syndrome (AIDS) (Schnittman and Fauci, 1994). Regulation of the immune system ensures host tissues are tolerated but this must be done without compromising necessary immune responses, otherwise this can result in chronic infection (Rosas-Taraco *et al*., 2012) or the suppression of anti-tumour responses (Wolf *et al*., 2003, Azuma *et al*., 2003). The opposite scenario results in autoimmunity (Wildin *et al.*, 2001, Bacchetta *et al.*, 2006, d'Hennezel *et al.*, 2009).

The mechanisms involved in preventing a breakdown of tolerance to self by

lymphocytes can be broadly divided into central and peripheral tolerance. In the case of T cells, central tolerance occurs in the thymus where first thymocytes (the precursors to T cells), which are capable of recognising self-major histocompatibility complex (MHC) molecules in the thymic cortex, are selected for further maturation in a process known as positive selection (Morris and Allen, 2013, Klein *et al.*, 2009). The thymocytes, which survive this process, are then subjected to negative selection in the medulla of the thymus, whereby cells with too high an affinity for self-MHC are deleted by apoptosis (Morris and Allen, 2013, Klein *et al.*, 2009). Tolerance to self is examined by the presentation of many tissue-specific antigens by medullary thymic epithelial cells, which is controlled by the transcription factor autoimmune regulator (AIRE) (Liston *et al.*, 2003). Dysfunction of AIRE in humans causes the autoimmune polyendocrine syndrome type I (Nagamine *et al.*, 1997).

Not all self-reactive T cells are deleted by central tolerance as a number of studies have identified the presence of self-antigen-specific conventional T cells (Tconv) in the peripheral blood of healthy individuals (Danke *et al.*, 2005, Viglietta *et al.*, 2002, Scholz *et al.*, 1998, Danke *et al.*, 2004). However, these cells are controlled once thymic egression has occurred by peripheral tolerance causing these cells to either be deleted, induced to become anergic (Greenwald *et al.*, 2001) or suppressed by Tregs (Sakaguchi *et al.*, 1995).

1.2 Regulatory T cells

There are several subsets of cells with regulatory properties: CD4+ CD25hi FOXP3+ Tregs (Sakaguchi *et al*., 1995, Baecher-Allan *et al*., 2001), type 1 regulatory (Tr1) cells (Groux *et al.*, 1997, Kemper *et al.*, 2003) (see Section 1.5) and T_H3 cells (Chen *et al.*, 1994). Also γδ T cells have also been shown to function in a regulatory manner in some environments (Peng *et al.*, 1996, Girardi *et al.*, 2002). The most examined are the CD4+ CD25hi FOXP3+ Tregs, which were studied in this thesis. Although these suppressor cells had been identified in the early 1970s (Gershon and Kondo, 1970, Gershon and Kondo, 1971) interest in this subset was only piqued after Sakaguchi and colleagues described the existence of murine CD4+ CD25+ T cells and their importance in preventing autoimmunity (Sakaguchi *et al.*, 1995). Their study demonstrated that depletion of these cells from immune cell suspensions injected into athymic mice led to autoimmunity, which could be prevented by the simultaneous administration of CD4+ CD25+ T cells alongside these injections (Sakaguchi *et al.*, 1995). Later termed Tregs, these cells were subsequently identified in humans (Baecher-Allan *et al.*, 2001, Jonuleit *et al.*, 2001, Taams *et al.*, 2001) and have since been implicated in several autoimmune diseases in terms of altered frequency (Morgan *et al.*, 2010) or function (Kriegel *et al.*, 2004, Lindley *et al.*, 2005, Viglietta *et al.*, 2004) (see Sections 1.8.4 and 1.14).

Tregs can be distinguished from other cell populations by the presence and level of expression of several markers. For example, both human and

murine Tregs express constitutively higher levels of CD25 compared to CD4+ Tconv (Sakaguchi et al., 2010). Tregs have also been shown to express the transcription factor forkhead box protein 3 (FoxP3 in mice and FOXP3 in humans) (Fontenot *et al.*, 2003, Fontenot *et al.*, 2005b), although in humans this is not specific to Tregs (Morgan *et al.*, 2005). Further investigation into both human and murine Tregs found they expressed low levels of CD127, whereas Tconv express high levels (Liu *et al.*, 2006b). The characteristic markers of Tregs are shown in Figure 1.1 and are discussed in detail in the following subsections.

1.2.1 CD25

CD25 is the non-signalling alpha-unit of the IL-2 receptor, also called IL-2RA, which together with CD122 and CD132 forms the high affinity IL-2 receptor (Malek and Bayer, 2004). It is constitutively expressed at high levels on Tregs (Baecher-Allan *et al.*, 2001), but upon activation both murine and human Tconv express CD25, albeit at lower levels than Tregs (Malek and Bayer, 2004). In humans CD4+ T cells have been found to express a spectrum of CD25 with *bone fide* Tregs expressing the top 1-2 % of this marker (Baecher-Allan *et al.*, 2001). IL-2 acting on Tregs further up-regulates this protein (Malek and Bayer, 2004), possibly through its maintenance of FOXP3 (Zorn *et al.*, 2006), which activates transcription of the IL-2RA gene (Wu *et al.*, 2006) (see Section 1.2.3). Selecting Tregs according to their high expression of CD25 is a commonly used strategy,

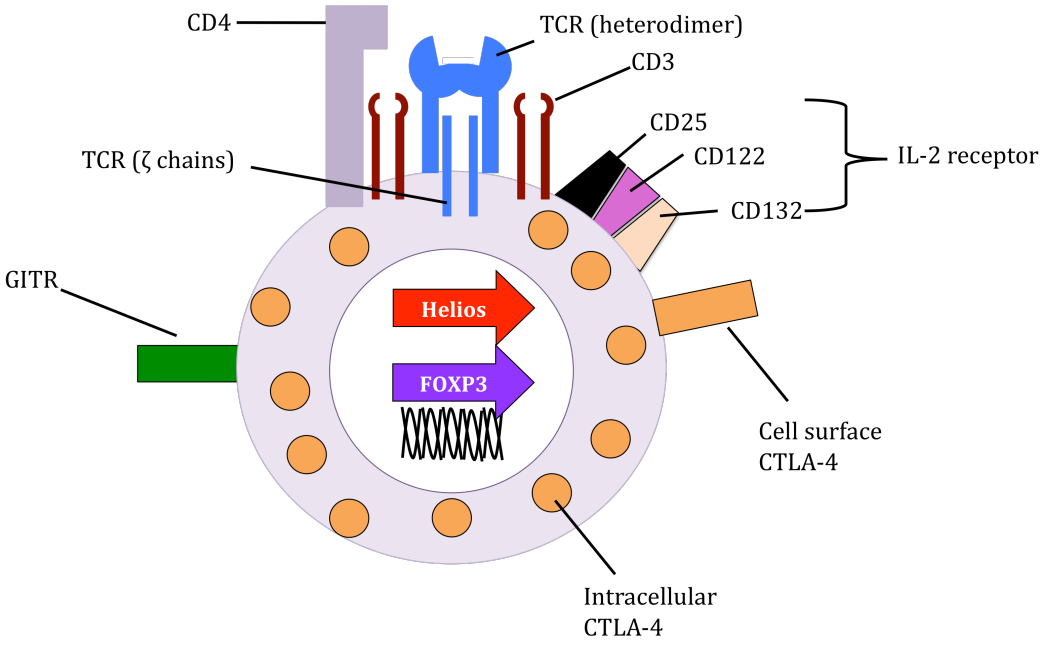

Figure 1.1 Characteristic markers of CD4+ CD25hi FOXP3+ Tregs

The above diagram shows the characteristic markers of CD4+ CD25hi FOXP3+ Tregs. The T-cell receptor (TCR) heterodimer interacts with antigen displayed by MHC molecules on antigen-presenting cells (APC); the ensuing signalling cascade involving the ζ chains and CD3 proteins of the TCR complex and the CD4 co-receptor. The suppressive action of Tregs requires activation of these cells. IL-2 is essential for the maintenance and function of Tregs and these cells constitutively display high levels of CD25, which together with CD122 and CD132, form the high affinity IL-2 receptor. There are several methods by which Tregs are reported to suppress including the involvement of GITR and CTLA-4. The transcription factor FOXP3 is believed to be essential for Tregs function, although the exact function of another transcription factor, Helios, has yet to be determined and it is still under debate as to whether Helios is unique to naturally-occurring Tregs.

although purer populations can be obtained when it is used in conjunction with low levels of CD127 (Liu *et al.*, 2006b) (see section below).

1.2.2 CD127

CD127 (IL-7RA) is the alpha unit of the IL-7 receptor that shares the same gamma chain (CD132) as the IL-2 receptor (Palmer *et al.*, 2008). It is expressed at high levels on resting Tconv (Malek and Bayer, 2004), but low levels on Tregs (Liu *et al.*, 2006b) from both mice and humans. IL-7 is required to maintain both naïve (Tan *et al.*, 2001) and memory Tconv (Dooms *et al.*, 2007, Li *et al.*, 2003), however CD127 is down-regulated upon activation (Dooms *et al.*, 2007). After the acute-phase response, CD127 is up-regulated again on Tconv via the action of IL-2 (Dooms *et al.*, 2007). FOXP3 has been shown to bind to the *IL-7RA* promoter and suppress transcription (Liu *et al.*, 2006b) (see section below). When Tregs are isolated according to high levels of CD25 expression but low levels of CD127, higher and purer numbers of these cells can be obtained (Liu *et al.*, 2006b). Therefore, this is currently the most common method of selecting these cells.

1.2.3 FOXP3

FOXP3 belongs to the forkhead/winged family of transcription factors (Bennett *et al.*, 2001). By examining αβ T cells from mice in which eGFP was

inserted into the first exon of the Foxp3 gene, this transcription factor was shown to be a marker of Tregs in mice (Fontenot *et al.*, 2005b). FOXP3 was later shown to be a marker of human Tregs also (Allan *et al.*, 2005) although unlike in mice, non-regulatory CD4+ T cells in humans transiently express FOXP3 upon activation (Allan *et al.*, 2005, Morgan *et al.*, 2005, Walker *et al.*, 2003). Mutations in the Foxp3 gene result in severe autoimmunity in both mice (Khattri *et al.*, 2003, Lahl *et al.*, 2007, Brunkow *et al.*, 2001) and humans (in whom this results in the immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX)-syndrome (see Section 1.8.1) (Wildin *et al.*, 2001, Bacchetta *et al.*, 2006, d'Hennezel *et al.*, 2009) highlighting its importance in Treg function. Indeed, ectopic expression of FoxP3 in murine CD4+ CD25- T cells has been reported to confer suppressive function (Fontenot *et al.*, 2003). However, this scenario in humans is more complex. Humans express two FOXP3 isoforms and if either or both of these isoforms are ectopically expressed in CD4+ naïve Tconv, the cells gain an anergic phenotype and do not produce IL-2 or interferon γ (IFNγ) (Allan *et al.*, 2005). Although human T cells transduced with both isoforms exhibit suppressive activity, this is much reduced compared to *bone fide* Tregs.

FOXP3, as part of a complex with other transcription factors, directly binds to the promoters of the IL-2, CTLA-4 (Wu *et al.*, 2006), IL-7 (Liu *et al.*, 2006b) and IL-2RA genes (Camperio *et al.*, 2012, Wu *et al.*, 2006) and either represses transcription (in the case of *IL-2* and *IL-7*) or activates

transcription (as for *CTLA-4* and *IL-2RA*). One study showed the characteristic gene silencing in murine Tregs mediated by FoxP3 involves its interaction with Ikaros transcription factor family member, Eos (Pan *et al.*, 2009); the direct interaction of these two molecules being essential for the Treg phenotype.

At present FOXP3 is one of the best markers available for selecting Tregs, providing the following examination procedures do not require these cells to be viable. The most efficient way of examining the purity of the Treg population is to determine the extent of methylation of the Treg-specific demethylated region (TSDR). The TSDR is located in the 5' un-translated region of the *Foxp3* locus and is rich in cytosine phosphate guanine (CpG) motifs (Floess *et al.*, 2007, Polansky *et al.*, 2008). In thymically-derived (or naturally occurring) Tregs (nTregs) (see Section 1.4), the CpG motifs are completely demethylated, however in Tconv and CD4+ CD25hi FOXP3+ Tregs induced in the periphery (iTregs) they are only partially methylated (Floess *et al.*, 2007, Polansky *et al.*, 2008).

1.2.4 Helios

Helios belongs to the Ikaros family of transcription factors and is thought to be a marker of nTregs (see Section 1.4) (Thornton *et al.*, 2010). However, it should be noted that others have found conflicting results with some studies showing that Helios can be expressed in murine and human iTregs both *in*

vitro and *in vivo* (Verhagen and Wraith, 2010, Getnet *et al.*, 2010, Akimova *et al.*, 2011). These studies argue that it is the manner in which these cells are activated rather than their origin that determines expression of Helios. The role of Helios is still unknown, although studies of murine and human T cells found that FOXP3 and Helios can interact (Getnet *et al.*, 2010, Thornton *et al.*, 2010). One study of mouse and human T cells showed that knock-down of FOXP3 or Helios has no affect on the other and depletion of Helios does not effect suppression (Thornton *et al.*, 2010), whilst another found that down-regulation of Helios in human T cells also down-regulated FOXP3 and decreased Treg suppression (Getnet *et al.*, 2010). A recent study demonstrated that both Helios and FoxP3 bind simultaneously to the *IL-2* promoter in murine Tregs and knock-down of Helios resulted in decreased binding of FoxP3 (Baine *et al.*, 2013). Further research is required to clarify the role of Helios in Tregs.

1.3 CD4+ CD25hi FOXP3+ Treg subpopulations

Tregs are not a homogenous population and can be divided into several different subpopulations depending on the markers selected (Miyara et al., 2009, Fletcher et al., 2009, Duhen et al., 2012). For example, Sakaguchi and colleagues demonstrated that three subpopulations exist based upon the expression of CD25 and either CD45RA or FOXP3 (Miyara *et al.*, 2009). Resting Tregs (rTregs) are CD25+ CD45RA+ FOXP3+, whilst activated Tregs (aTregs) are CD25hi CD45RA- FOXP3hi. A third population described by

Miyara *et al.* (2009) was a non-suppressive CD25+ CD45RA- FOXP3+ IL-17-producing population. However, work by others suggests that this latter population does in fact contain a suppressive population of Tregs (Scotta *et al.*, 2012), since termed memory Tregs (mTregs) and are referred to as such in this thesis. rTregs are CD31+ suggesting they are recent thymic emigrants and upon activation these cells give rise to aTregs, whilst the original aTreg population suppresses upon activation and subsequently undergoes apoptosis (Miyara *et al.*, 2009). Whether rTregs differentiate into mTregs is yet to be investigated. Also, recent work has identified four subpopulations of memory Tregs, which mirror T-helper cells (T_H1-, T_H2-, T_H17- and T_H22-like Tregs) in terms of their characteristic transcription factors and cytokine profiles (Duhen *et al.*, 2012). These cells still exhibit suppressive function and in the case of T_H1- and T_H17-like Tregs, also produce the immunosuppressive cytokine, IL-10.

1.4 Thymic generation of naturally-occurring Tregs

CD4+ CD25hi FOXP3+ Tregs can either be thymically-derived (nTregs) or induced in the periphery (iTregs) (Bluestone and Abbas, 2003). Evidence nTregs are produced in the thymus was shown by a study of BALB/c mice, which demonstrated the presence of CD4+ CD25+ thymocytes in the thymi but not in the periphery of two-day-old mice (Itoh *et al.*, 1999). This study also isolated CD4- CD8- Thy1.2+ thymocytes from BALB/c mice and inserted these directly into the thymi of other BALB/c mice expressing the Thy1.1

antigen. One week later, a significant number of CD4+ CD25+ thymocytes were Thy1.2+, thus indicating they had arisen from donor thymocytes and had been generated in the thymi of the recipients (Itoh *et al.*, 1999). In humans, a study of children with DiGeorge syndrome (characterised by inherited thymic hypoplasia) demonstrated that compared to age-matched controls individuals with this syndrome have significantly lower frequencies and absolute levels of CD4+ CD25hi T cells suggesting nTregs are produced within the thymus in humans also (Sullivan *et al.*, 2002).

Whilst it is now appreciated that nTregs originate in the thymus, the actual process of generation is still a matter of controversy. It appears that although precursors to Tregs are subject to the same central tolerance mechanisms as CD4+ CD25- FoxP3- thymocytes (see Section 1.1), the latter are produced at a much faster rate than the Treg precursors (Fontenot *et al.*, 2005a). One of the current theories is that a two-step mechanism takes place in the thymus to produce these cells (Lio and Hsieh, 2008, Burchill *et al.*, 2008) explaining this lag period in Treg generation (Hsieh *et al.*, 2012). This model suggests that Tregs are generated from CD4+ CD8- single positive thymocytes (Hsieh *et al.*, 2012, Burchill *et al.*, 2008, Lio and Hsieh, 2008) and the first stage in this process occurs between positive and negative selection and is T-cell receptor (TCR)-dependent (Burchill *et al.*, 2008, Lio and Hsieh, 2008). TCR interaction with MHC class II molecules presenting self-antigen must occur at the same time as CD28 co-stimulation to up-regulate CD25 (Tai *et al.*, 2005, Bautista *et al.*, 2009), which sensitises

these cells to IL-2 signalling in the second stage, which is TCR-independent. The phosphorylation of the transcription factor, signal transducer and activator of transcription 5a (STAT5a) occurs in the IL-2 signalling pathway and its over-expression has been demonstrated to increase the frequency of nTregs (Burchill *et al.*, 2008). This suggests IL-2 signalling rescues these cells from undergoing negative selection, which would otherwise occur, as well as up-regulating FoxP3 (Burchill *et al.*, 2008).

Another matter of controversy is the affinity with which these Treg precursors recognise their cognate antigens. Although it may appear logical that these thymocytes have a relatively low affinity for their antigens thus allowing them to escape from negative selection, it has been demonstrated by some that a high affinity for self-peptide is required for the generation of Tregs (Jordan *et al.*, 2001). This research is impeded by the fact that using monoclonal TCR transgenic mice actually decreases the thymic yield of Tregs, as the increased competition that arises in the thymus containing TCRs exhibiting the same affinity favours development of Tconv but not Tregs (Bautista *et al.*, 2009). These findings gave rise the 'niche hypothesis' which states that only thymocytes recognising rare self-antigens will be produced (Bautista *et al.*, 2009, Hsieh *et al.*, 2012). Indeed it has been shown that Tregs recognising ubiquitous self-antigen are deleted and only those recognising tissue-specific antigens are released from the thymus (Bensinger *et al.*, 2001).

Contradictory to the 'niche hypothesis' is the 'buddy hypothesis' which states that as nTregs are needed to suppress autoreactive Tconv responses in the periphery, both of these cell types would express the same TCR (Hsieh *et al.*, 2012). However if there is an increase in the number of thymocytes recognising the same antigen this will increase competition, thus decreasing thymic Treg output (Bautista *et al.*, 2009). In support of the argument against this, studies have shown that the level of overlap of TCR repertoires between Tregs with both naïve and memory Tconv was very small (~5%) (Burchill *et al.*, 2008, Lio and Hsieh, 2008).

1.5 Tr1 cells

Tr1 cells produce high levels of IL-10 with equal or higher levels of IFNγ (Groux *et al.*, 1997, Kemper *et al.*, 2003). These cells have to date only been studied *in vitro* (although others have reported cells with Tr1 characteristics when studied *ex vivo*. See Chapter Seven, Section 7.3.1 for a more detailed description) and there are several ways in which these cells can be generated from CD4+ Tconv. One such method is by the simultaneous activation of CD3 together with the complement receptor CD46 in an IL-2-dependent manner (Kemper *et al.*, 2003, Cardone *et al.*, 2010). First identified for its roles in binding and degrading the opsonins C3b and C4b (Cole *et al.*, 1985, Cardone *et al.*, 2010, Seya *et al.*, 1986) CD46 is a type I transmembrane glycoprotein, of which there are four different isoforms, each possessing one of two cytoplasmic domains designated CYT-1 and CYT-

2 (Cardone *et al.*, 2010). Although both CYT-1 and CYT-2 can both bind to the Ste20-related proline alanine-rich kinase (SPAK) only its interaction with CYT-1 can induce IL-10 production via the phosphorylation of the kinase Erk when in the presence of high IL-2 concentrations (Cardone et al., 2010). Activation of CD3 and CD46 also causes the up-regulation of the transcriptional regulators ICER and CREM, which in the presence of high IL-2 concentrations, translocate to the nucleus and inhibit IL-2 transcription (Cardone et al., 2010). Interestingly an IPEX-like syndrome (see Section 1.8.1) has been reported in one individual who was found to have a deficiency in CD25 expression (Caudy *et al.*, 2007). This individual had CD4+ T cells that could not be induced to produce IL-10 when cultured *ex vivo* with anti-CD3/46 antibodies, highlighting the requirement of CD25 for the induction of Tr1-like cells.

The markers for Tr1 cells are still unclear and it is thought they do not express FOXP3 (Apetoh *et al.*, 2010). Therefore, these cells are generally characterised by their production of cytokines. The mechanism(s) by which Tr1 cells suppress are also not completely understood, but they are thought to implement an IL-10-dependent mechanism (Kemper *et al.*, 2003, Groux *et al.*, 1997) and can also destroy antigen-presenting cells (APC) by perforin and granzyme B (Grossman *et al.*, 2004). Although IFNγ has pro-inflammatory effects, it has been reported that this cytokine inhibits the action of T_H17 cells (Apetoh *et al.*, 2010).

The study by Cardone *et al.* (2010) showed that at low concentrations of IL-2 (less than 5 IU/ml) IFNγ-producing T_H1 cells were generated, but at higher IL-2 concentrations Tr1 cells were induced. The authors refer to this as the 'T_H1 cell to Tr1 cell switch' and suggest that during an immune response CD46 on T_H1 cells becomes activated promoting the production of IFNγ, but as IL-2 levels rise the level of this cytokine decreases and IL-10 is up-regulated. Indeed, they have shown that activated CD4+ T cells produced the complement protein C3b and were themselves coated with this complement protein, which activates CD46, thus promoting tolerance and protecting surrounding tissues (Cardone *et al.*, 2010, Cope *et al.*, 2011).

1.6 Mechanisms of CD4+ CD25hi FOXP3+ Treg suppression

The mechanisms by which Tregs suppress are not fully understood, although it appears there are several ways by which they can do so. For example, Tregs constitutively express cytotoxic lymphocyte antigen-4 (CTLA-4; CD152) (Tsaknaridis *et al.*, 2003), which binds to CD80/86 with a higher affinity than CD28 (Linsley *et al.*, 1991, Wicker *et al.*, 2005) initiating a negative feedback mechanism leading to the cessation of TCR signalling (Linsley *et al.*, 1991, Valk *et al.*, 2008). Tregs have been shown to down-regulate CD80/CD86 on APC in a CTLA-4-dependent manner (Oderup *et al.*, 2006, Onishi *et al.*, 2008, Wing *et al.*, 2008), later demonstrated to be due to trans-endocytosis (Qureshi *et al.*, 2011). Also, CD80/86 ligation by CTLA-4 has been shown to cause indoleamine 2,3-dioxygenase (IDO) production in

dendritic cells (DCs), which catabolises tryptophan and consequently inhibits T cell proliferation (Munn *et al.*, 2004, Hwu *et al.*, 2000, Munn *et al.*, 2002) (see Figure 1.2a).

Murine Tregs and a subset of human Tregs constitutively express the enzyme CD39 on their cell surface (Borsellino *et al.*, 2007, Fletcher *et al.*, 2009). CD39 catabolises extracellular adenosine triphosphate (ATP) and adenosine diphosphate (ADP) to adenosine monophosphate (AMP) (Borsellino *et al.*, 2007), which is further catabolised by CD73 to extracellular adenosine (Deaglio *et al.*, 2007). This mechanism causes suppression by the depletion of extracellular ATP, but also extracellular adenosine causes the accumulation of intracellular cyclic AMP (cAMP), resulting in the accumulation of IL-10 (Ernst *et al.*, 2010) and suppression of IL-2 transcription (Bopp *et al.*, 2007) (see Figure 1.2b).

Suppression can also be caused by apoptosis of the target cell (Cao *et al.*, 2007, Gondek *et al.*, 2005). Granzyme B is a serine protease, which acts as a toxin used by the immune system to kill target cells (Wowk and Trapani, 2004). Granzyme B has been shown to cleave caspases, initiating a cascade ultimately resulting in apoptosis (Wowk and Trapani, 2004). Granzyme B in humans is usually associated with suppression by Tr1 cells (Grossman *et al.*, 2004) (see Section 1.5) although one study reported that Tregs do express granzyme B (Bryl *et al.*, 2009) (see Figure 1.2c).

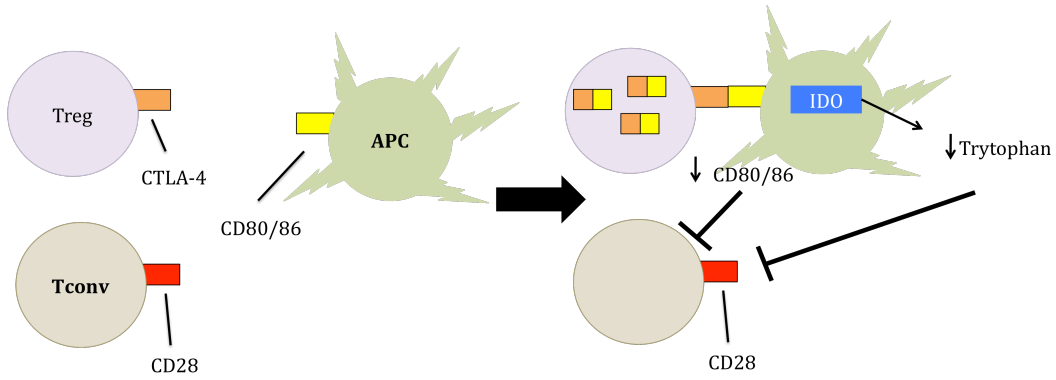

Figure 1.2a Suppression mediated by CTLA-4

Shown here are the suppressive mechanisms mediated by CTLA-4. CTLA-4-dependent trans-endocytosis causes down-regulation of CD80/86 on APC; the lack of these CD28 agonists causing Tconv suppression. Also, CTLA-4:CD80/86 ligation causes the production of IDO, resulting in tryptophan depletion.

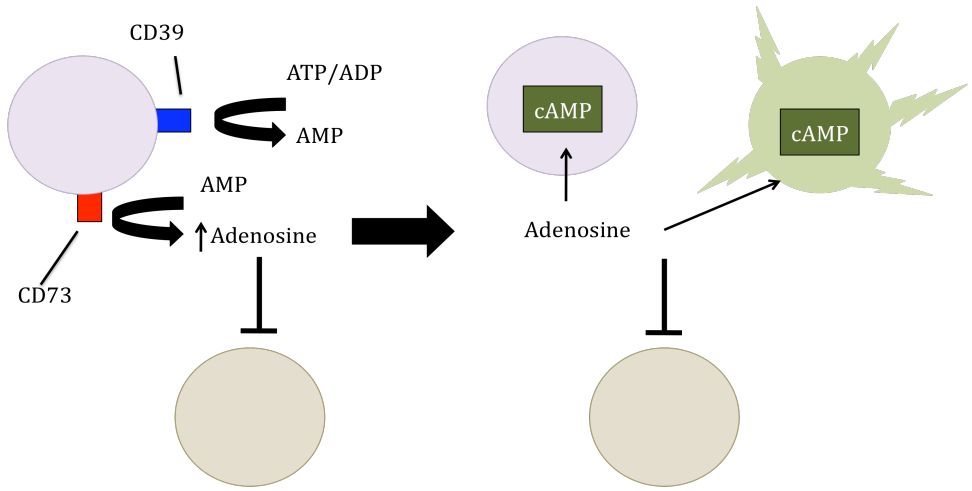

Figure 1.2b Suppression via depletion of extracellular ATP and accumulation of intracellular cAMP

Extracellular ATP and ADP is catabolised first to AMP and then to extracellular adenosine, by CD39 and CD73, respectively. Adenosine causes the accumulation of cAMP in Tregs and APC also causing suppression.

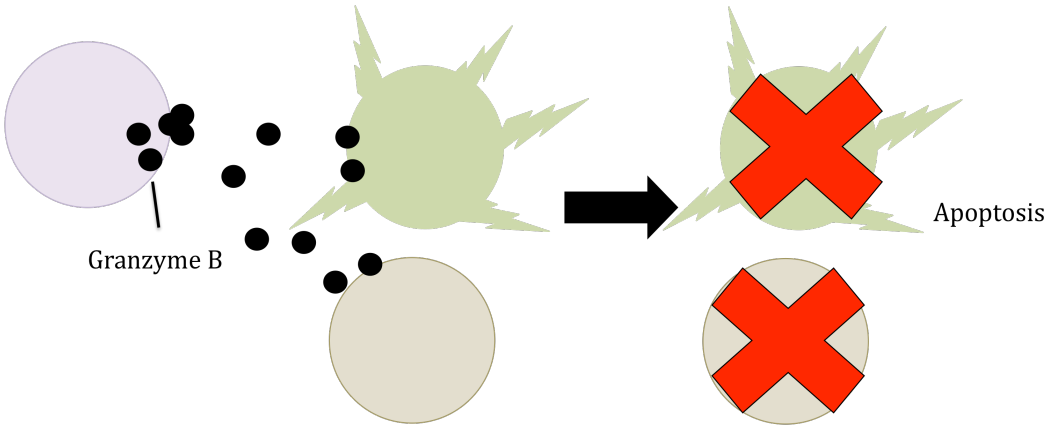

Figure 1.2c Granzyme B causes apoptosis in target cells resulting in suppression

Granzyme B production by Tregs causes apoptosis in target cells, ultimately causing suppression.

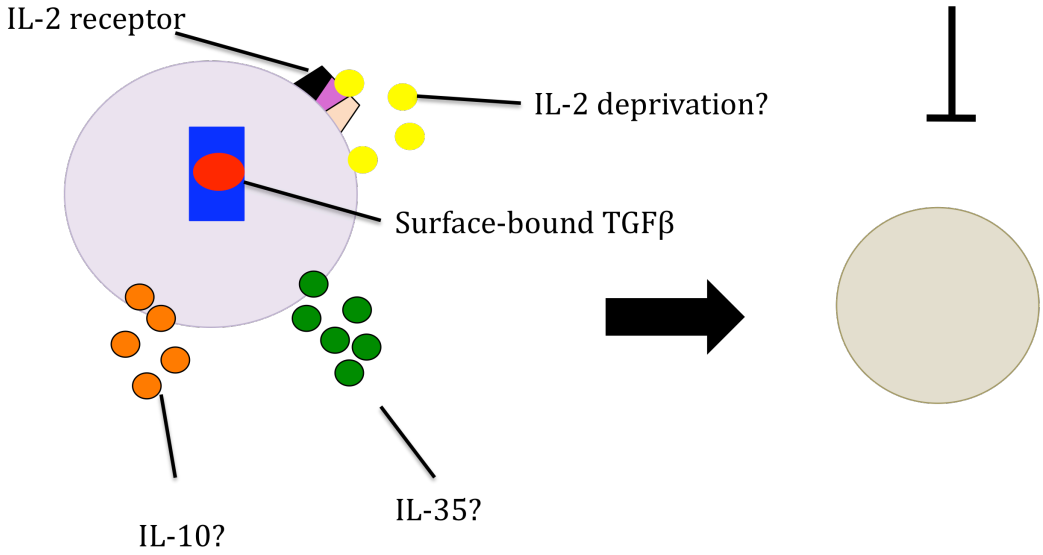

Figure 1.2d Cytokine-mediated suppression

Cell-surface-bound TGFβ mediates suppression via as yet unknown mechanisms. It is currently unclear as to whether IL-10, IL-35 or IL-2 deprivation are also mechanisms of suppression.

Tregs have also been reported to suppress via various cytokines such as cell-surface-bound transforming growth factor β (TGFβ) (Nakamura *et al.*, 2001, Nakamura *et al.*, 2004). However, there are conflicting reports as to whether they can also suppress by IL-10 (Joetham *et al.*, 2007, Asseman *et al.*, 1999, Takahashi *et al.*, 1998, Taams *et al.*, 2001, Ng *et al.*, 2001, Ehrenstein *et al.*, 2004) or IL-35 (Bardel *et al.*, 2008, Chaturvedi *et al.*, 2011, Collison *et al.*, 2007). Studies have also reported the suppression of murine Tconv by IL-2 deprivation caused by Tregs (Pandiyan *et al.*, 2007, Wang *et al.*, 2010). However, one study of human Tregs reported that these cells did not suppress using this mechanism, (Tran *et al.*, 2009), although it may be possible that other suppression mechanisms were involved as a compensatory measure (see Figure 1.2d).

1.7 Mechanisms of resistance to Treg suppression

To be able to mount an effective immune response against pathogens Tconv must be able to acquire resistance to Treg suppression. Indeed the factors which cause increased resistance are all associated with infection and inflammation (Walker, 2009). A model of 'tuned suppression' has been proposed, whereby during an infection the suppressive function of Tregs is not diminished but instead increased in the presence of factors associated with such an immune response, although Tconv are resistant to their action (Walker, 2009). In this model, Tregs respond to the same factors, which cause Tconv to become refractory and the increased IL-2 produced by Tconv

maintains the presence and function of Tregs. Thus when the infection subsides, Tconv once again become sensitive to the Tregs they have helped to sustain during the infection.

Several studies of murine and human Tregs have found that increasing the TCR signal given to Treg-Tconv *in vitro* co-cultures raised Tconv proliferation and reduced suppression (Takahashi *et al.*, 1998, Thornton and Shevach, 1998, Baecher-Allan *et al.*, 2001, Baecher-Allan *et al.*, 2002, Viglietta *et al.*, 2004, Lindley *et al.*, 2005). At very strong signals, Treg anergy was also overcome (Thornton and Shevach, 1998, Baecher-Allan *et al.*, 2001, Baecher-Allan *et al.*, 2002). One study mimicked the effect of TCR signalling by adding ionomycin to murine Tconv to enhance the intracellular calcium flux and found *in vitro* suppression was also abrogated (Wang *et al.*, 2010). Further investigation was conducted, whereby the Tconv and Treg populations were activated separately *in vitro* with a range of stimuli strengths before being placed into co-culture (Baecher-Allan *et al.*, 2002). This study showed the stronger the stimulus used to pre-activate the Tconv the quicker these cells became refractory to Treg suppression. It has been suggested that suppression is abrogated at high signal strengths as self-antigens are thought to stimulate cells weakly, whereas foreign antigens stimulate cells stronger and inhibition of an immune response against the latter is undesirable (Baecher-Allan and Hafler, 2006, Walker and Abbas, 2002, Bouneaud *et al.*, 2000).

Addition of co-stimulation in the form of anti-CD28 antibody has also been reported to abrogate suppression of Tconv proliferation when added at high concentrations (Takahashi *et al.*, 1998, Thornton and Shevach, 1998, Baecher-Allan *et al.*, 2001, Baecher-Allan *et al.*, 2002). These studies all examined CD25- Tconv, which are predominately naïve Tconv requiring co-stimulation, via ligation of CD28 to CD80/86 on APC to become activated (Croft *et al.*, 1994). It has been speculated that when presenting self-antigen, DCs remain immature or become tolerogenic and thus display low levels of CD80 and CD86 (Walker and Abbas, 2002). Conversely, foreign antigen presentation is often combined with activation of Toll-like receptors (TLRs) leading to the maturation of DCs with up-regulation of CD80 and CD86. Hence explaining why strong co-stimulation also leads to inhibition of suppression.

Other factors, which influence resistance, include TLR engagement, with several studies showing the ligation of TLRs allowed Tconv to proliferate even in the presence of Tregs (Pasare and Medzhitov, 2003, Crellin *et al.*, 2005, Liu *et al.*, 2006a). Certain cytokines also confer resistance, such as IL-6 (Pasare and Medzhitov, 2003); TNF-α (Yang *et al.*, 1994, Wu *et al.*, 2002) and whilst those which signal through the common gamma chain (CD132) maintain Tregs (Thornton *et al.*, 2004), they also inhibit Treg function (Walker, 2009), supporting the notion of 'tuned suppression' described above. In addition, the glucocorticoid-induced tumour necrosis factor receptor-related protein (GITR) ligand also abrogates suppression (McHugh

et al., 2002, Stephens *et al*., 2004, Shimizu *et al*., 2002).

1.8 The role of Tregs in disease

1.8.1 IPEX

The pivotal role of Tregs in preventing autoimmunity has been clearly demonstrated in mouse models. A deficiency in the number or function of these cells results in uncontrolled lymphoproliferation and autoimmune disease (Sakaguchi *et al.*, 1995, Kramer *et al.*, 1995, Khattri *et al.*, 2003), an example being the Scurfy mouse, which lacks Tregs due to a frame-shift mutation in the Foxp3 gene (Khattri *et al.*, 2003, Lahl *et al.*, 2007, Brunkow *et al.*, 2001). Likewise in humans, mutations in the *FOXP3* gene cause the IPEX-syndrome (Bacchetta *et al.*, 2006, Wildin *et al.*, 2001, d'Hennezel *et al.*, 2009, Le Bras and Geha, 2006, Chatila *et al.*, 2000) a rare disorder, characterised by the early-onset of a number of autoimmune and allergic conditions (Levy-Lahad and Wildin, 2001).

Fewer than 150 individuals worldwide have been diagnosed with IPEX and to date around sixty different mutations in the FOXP3 gene have been associated with the disease (Barzaghi et al., 2012). Onset of this disease usually occurs with the first few months of birth and patients are not expected to live beyond the first two years of life unless treated with a bone marrow transplant (Barzaghi et al., 2012). The majority of sufferers develop T1D (see Section 1.9) shortly after IPEX onset (Wildin et al., 2001, van der

Vliet and Nieuwenhuis, 2007, Barzaghi et al., 2012) and similar to polygenic T1D, insulitis and beta-cell loss in the pancreas have been reported in such individuals (see Section 1.9) (Wildin et al., 2002). It has been suggested that the site of the mutation in the FOXP3 gene is linked to the severity of the disease, as several FOXP3 mutations do not prevent this transcription factor from being expressed by CD4+ CD25+ T cells in IPEX patients (Bacchetta et al., 2006, d'Hennezel et al., 2009), although this hypothesis cannot be directly examined as the rarity of this condition hinders the recruitment of a sufficient number of patients to analyse this. However, studies have shown that IPEX patients who express FOXP3 have similar frequencies of CD4+ CD25hi FOXP3+ Tregs to healthy individuals, but in IPEX patients these cells are highly deficient in function (Bacchetta et al., 2006, d'Hennezel et al., 2009). It is possible that whilst these FOXP3 mutations do not prevent the expression of FOXP3 they may prevent the interaction of FOXP3 with its binding partners, such as Eos (Pan et al., 2009) and Helios (Thornton et al., 2010, Getnet et al., 2010) (see Sections 1.2.3 and 1.2.4) abrogating the suppressive function of Tregs.

1.8.2 Suppression of anti-tumour responses

Due to the tolerogenic nature of Tregs in preventing autoreactive responses, it is perhaps unsurprising that they are thought to inhibit anti-tumour immune responses. An increase in Treg frequency in peripheral blood and their presence among tumour-infiltrating lymphocytes has been reported in

several types of cancer (Ichihara *et al.*, 2003, Liyanage *et al.*, 2002, Marshall *et al.*, 2004, Woo *et al.*, 2002, Ormandy *et al.*, 2005). Also, Tregs can suppress the cytotoxic functions of natural killer (NK) cells (Wolf *et al.*, 2003) and NKT cells (Azuma *et al.*, 2003), at least *in vitro*; cell populations involved in immune responses against tumours.

1.8.3 Allergies

Several studies have shown Tconv recognising non-harmful foreign antigens such as those present in cat dander (Ling *et al.*, 2004), milk (Karlsson *et al.*, 2004), grass pollen (Ling *et al.*, 2004) and birch pollen (Grindebacke *et al.*, 2004) are present in both allergic and non-allergic individuals. Tregs from non-allergic subjects were able to suppress antigen-specific responses to a significantly higher degree than those from allergic volunteers (Ling *et al.*, 2004, Grindebacke *et al.*, 2004, Cavani *et al.*, 2003). This suggests Tregs are defective in function in allergic individuals.

1.8.4 Autoimmunity

Both individuals with and without an autoimmune disease possess autoreactive Tconv (Danke *et al.*, 2004, Danke *et al.*, 2005, Viglietta *et al.*, 2002, Scholz *et al.*, 1998) recognising autoantigens associated with T1D (Viglietta *et al.*, 2002, Danke *et al.*, 2004, Danke *et al.*, 2005) and relapsing-remitting multiple sclerosis (RRMS) (Scholz *et al.*, 1998). However, these cells are of a naïve phenotype, whereas the majority of those from

autoimmune patients tend to possess a memory phenotype (Viglietta *et al.*, 2002, Danke *et al.*, 2005, Scholz *et al.*, 1998), suggesting these cells have been previously activated and are implicated in the destruction of host tissue. The autoreactive Tconv from healthy donors may therefore be under tighter regulatory control.

A decreased frequency of Tregs in peripheral blood has been shown in some autoimmune diseases, such as Wegener's granulomatosis (Morgan *et al.*, 2010) and Bechet's disease (Kim *et al.*, 2012), whilst contradictory results have been reported for others, such as systemic lupus erythematosus (SLE) (Liu *et al.*, 2004, Kim *et al.*, 2012) and rheumatoid arthritis (RA) (Kim *et al.*, 2012, van Amelsfort *et al.*, 2004). However, later studies tend to include more recently identified markers to select a purer Treg population, possibly explaining these contradictory findings. The most recent study selected Tregs according to the presence of CD4, FOXP3 and high levels of CD25 and found no difference in Treg frequency in SLE and RA (Kim *et al.*, 2012). When the Treg subpopulations rTreg, mTreg and aTreg (Section 1.3) were examined, aTregs were found to be lower in RA patients and patients with Bechet's Disease (Kim *et al.*, 2012). Also RA donors showed a significantly lower aTreg:rTreg ratio than controls (Kim *et al.*, 2012). Higher levels of rTregs and mTregs and lower levels of aTregs have also been reported in active SLE (Miyara *et al.*, 2009).

Whilst no difference in Treg frequency has been reported for autoimmune

polyendocrine syndrome type 2 (APS II) (Kriegel *et al.*, 2004), RRMS (Fletcher *et al.*, 2009, Viglietta *et al.*, 2004) and T1D (Liu *et al.*, 2006b, Lindley *et al.*, 2005, Lawson *et al.*, 2008, Brusko *et al.*, 2005, Putnam *et al.*, 2005), these autoimmune diseases do show defective Treg function (A full description of the role of Tregs in T1D is given in Section 1.14). A defect was also identified in Treg function in active (but not inactive) SLE (Valencia *et al.*, 2007). It should be noted though that generally studies in humans only examine Tregs from the peripheral blood and not from the affected tissues. Therefore, Tregs in these tissues may or may not show the same deficiencies or defects as those in peripheral blood. Indeed, in RA no functional defect was seen in peripheral blood Tregs, but those from the synovial fluid showed enhanced suppression (van Amelsfort *et al.*, 2004). In addition to suppression of Tconv proliferation, a defect in suppression of IFNγ production by Tconv has been shown in RRMS (Viglietta *et al.*, 2004), active SLE (Valencia *et al.*, 2007) and ROT1D (Lindley *et al.*, 2005). Interestingly, in RA although Treg suppression of Tconv proliferation is not defective (van Amelsfort *et al.*, 2004, Ehrenstein *et al.*, 2004) the suppression of IFNγ and TNFα production by Tconv is (Ehrenstein *et al.*, 2004).

This defect in Treg suppression may be due to a decreased suppressive capacity of the Tregs themselves or it might result from an increased resistance of Tconv to suppression, or a combination of both of these factors. A number of studies have investigated this by co-culturing Tregs from

autoimmune individuals with Tconv from healthy individuals and *vice versa* (crossover suppression assays, see Chapter Six, Section 6.2.8). From these studies, a defect in the Treg population itself was revealed in patients with APS II (Kriegel *et al.*, 2004), RRMS (Viglietta *et al.*, 2004) and active SLE (Valencia *et al.*, 2007). Also, the defect in cytokine suppression in RA was shown to arise from a defect in Tregs (Ehrenstein *et al.*, 2004). These findings differ from those found in T1D (Lawson *et al.*, 2008, Schneider *et al.*, 2008) (see Section 1.14).

Although several allergies and autoimmune conditions exhibit deficient Treg function, allergies are a T_H2-mediated condition (Okada et al., 2010) whereas T_H1 cells are implicated in autoimmune conditions, including T1D (Lehuen et al., 2010). Although T1D patients are at an increased risk of developing other autoimmune conditions, such as Addison's Disease (Lewis, 2011) an inverse correlation between T1D and development of allergies has been reported (Engkilde et al., 2006). Further research is required to resolve this apparent discrepancy.

1.9 Type 1A Diabetes

The term Diabetes Mellitus encompasses a range of heterogeneous metabolic disorders all characterised by hyperglycaemia, ensuing from insufficient insulin action (Gavin *et al.*, 1997). Unlike Type 2 Diabetes, which largely results from a resistance of target cells to insulin function (Patel and

Macerollo, 2010), T1D is caused by the destruction of the cells which produce this hormone; the beta-cells in the islets of Langerhans situated within the pancreas (Gepts, 1965, Banting and Best, 1990). It should be noted here that T1D can be subdivided into the autoimmune disease Type 1A Diabetes, the subject of this thesis (and in which it is referred to as T1D), and Type 1B Diabetes, an idiopathic disease, which will not be discussed further (Gavin *et al.*, 1997).

At diagnosis, T1D patients display a number of symptoms including; polyphagia, polydipsia, polyuria, glucosuria, weight loss and in many cases ketoacidosis (Gavin *et al.*, 2003). Ketoacidosis results in coma and death if left untreated. Prior to the discovery and subsequent development of insulin therapy, T1D was invariably fatal (Joslin, 1916, Banting *et al.*, 1922, Banting and Best, 1990, Joslin, 1924). Although a means to prevent hyperglycaemia, improve the quality of life and ultimately vastly prolong survival, a life-long dependency on insulin is by no means a cure. T1D patients are at risk of developing microvascular (retinopathy, nephropathy and neuropathy) and macrovascular (cardiovascular disease and stroke) complications (Daneman, 2006). Their life-expectancy is also shortened by around twenty years (Daneman, 2006).

T1D is increasing worldwide, with striking increases seen in countries with previously low incidence rates (Gale, 2002). It was estimated at the end of 2012 that there were around 435,000 people with T1D in the U.K. and

around 55 million worldwide (D.U.K., 2012). The number in the U.K. is predicted to rise to 750,000 by 2025 (D.U.K., 2012). Aside from the obvious impact on human suffering, these are sobering facts for global economies. In the U.K. alone, T1D was estimated to cost the NHS in England and Wales £175 million between 2010-2011 (Kruger and Brennan, 2012).

1.10 T1D is an autoimmune disease

T1D is an autoimmune disease as evidenced by the presence of insulitis (infiltration of leukocytes into the pancreatic islets) at onset (Foulis *et al.*, 1986). Also immunosuppressants have been shown to slow progress of the disease (Feutren *et al.*, 1986, Carel *et al.*, 1996, Piemonti *et al.*, 2011, Herold *et al.*, 2012) and many of the genes predisposing to this disorder function within the immune system (Todd, 2010, Vella *et al.*, 2005, Cucca *et al.*, 2001, Erlich *et al.*, 2008, Ueda *et al.*, 2003, Vafiadis *et al.*, 1997) (see Section 1.13). Furthermore, prior to its classification as an autoimmune disease, one report demonstrated that this disease could be transferred via a bone marrow transplant between a diabetic donor and their non-diabetic sibling recipient (Lampeter *et al.*, 1993). Finally, as discussed in Section 1.8.4**,** islet-autoreactive T cells, although present in the peripheral blood of both non-diabetics and diabetics, are mainly of a memory phenotype in individuals with T1D (Danke *et al.*, 2004, Danke *et al.*, 2005, Viglietta *et al.*, 2002).

1.11 Beta-cell destruction

Autoreactive CD8+ T cells, recognising islet-autoantigens have been shown to kill human beta-cell lines *ex vivo* (Skowera *et al.*, 2008, Bulek *et al.*, 2012). In the case of CD8+ Tconv recognising pre-pro-insulin (PPI), the higher the glucose concentration, the more PPI these cells produce, thus increasing the rate of beta-cell destruction (Skowera *et al.*, 2008). However, the destruction of beta-cells *in vivo* is believed to be a much more complex process involving many different leukocyte populations and a myriad of pro-apoptotic mechanisms.

Beta-cells appear to play a role in their destruction by the secretion of chemokines, such as CCL2, which recruits macrophages to the vicinity (Lehuen *et al.*, 2010). Macrophages produce TNFα, reactive oxygen species (ROS) and IL-1β (the receptor of which is expressed at high levels on the beta-cell surface) all of which are implicated in beta-cell destruction (Lehuen *et al.*, 2010). Indeed, *in vitro* incubation of rat beta-cells with IL-1β and IFNγ led to the activation of the transcription factor nuclear factor kappa β (NF-κB), which initiates a signalling cascade resulting in beta-cell death (Cnop *et al.*, 2005). Several phagocyte populations are also recruited to the islets and produce IFNγ, which has a number of other effects upon the beta-cells (Cnop *et al.*, 2005). For instance, IFNγ causes the up-regulation of the pro-apoptotic protein, death protein 5 (Moore *et al.*, 2010) and CD95, making beta-cells susceptible to apoptosis if they encounter activated

CD95L-expressing T cells (Lehuen *et al.*, 2010); up-regulation of MHC class I molecules on the surface of beta-cells, thus exacerbating destruction by CD8+ Tconv (Cnop *et al.*, 2005), which also produce IFNγ themselves (Lehuen *et al.*, 2010) and causes beta-cells to secrete CXCL10, leading to the recruitment of T cells, NK cells, macrophages and DCs (Lehuen *et al.*, 2010). CD4 Tconv produce CCL1, also recruiting macrophages to the site of beta-cell destruction (Lehuen *et al.*, 2010). Another cytokine produced by macrophages is IL-12, involved in the differentiation of CD8+ cytotoxic T lymphocytes (CTLs) (Lehuen *et al.*, 2010). These findings are summarised in Figure 1.3.

Interestingly, residual beta-cell function has been shown in T1D patients who have had the disease for more than fifty years (Keenan *et al.*, 2010). Higher levels of insulin production and beta-cell presence was linked with an older age at onset. Also, despite beta-cell apoptosis there was also evidence of beta-cell proliferation.

1.12 Animal models of T1D

A substantial quantity of the research conducted in T1D involves the use of animal models. These allow researchers to investigate the aetiology, pathology and possible treatments of the disease. Discussed in this section are the most commonly used T1D animal models.

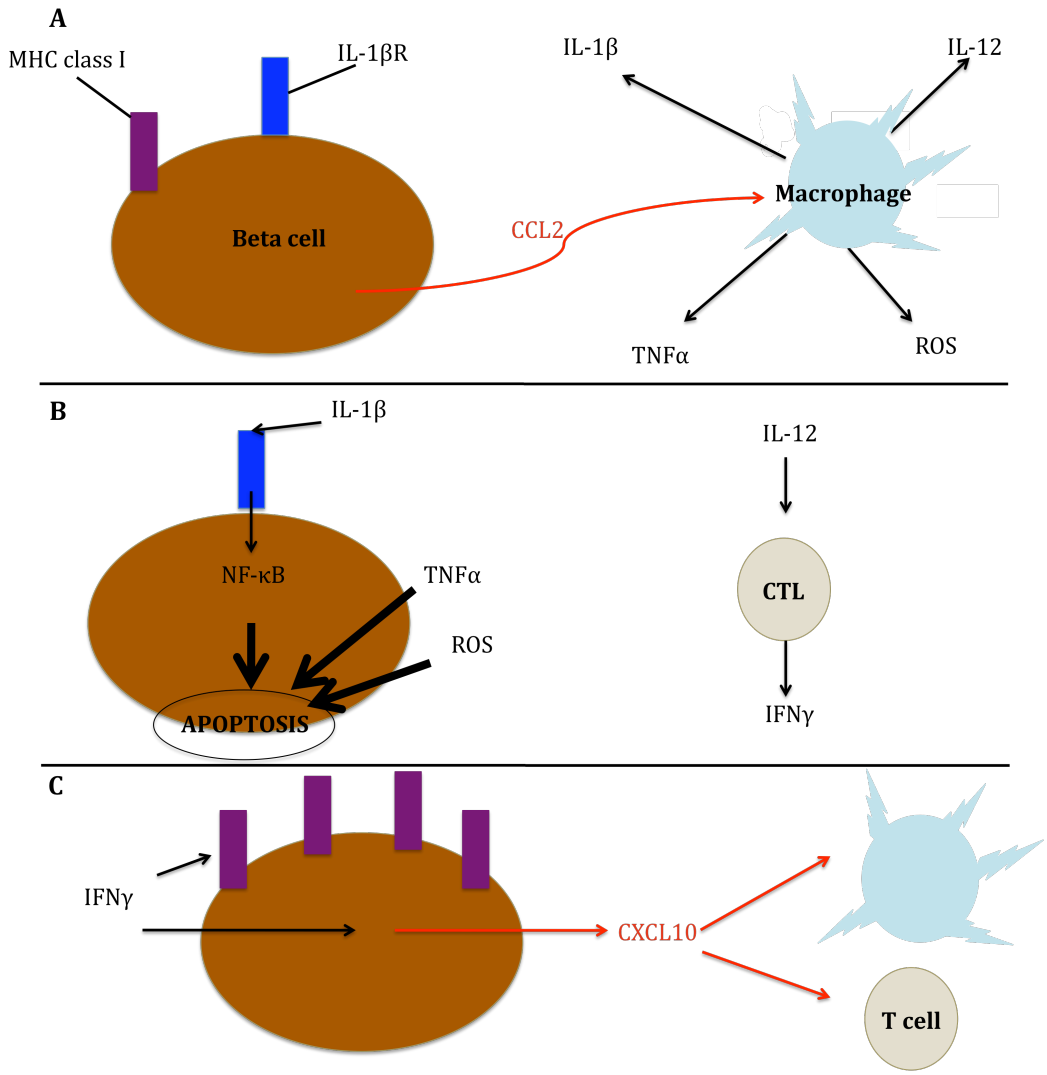

Figure 1.3 Summarised diagram of beta-cell destruction

Beta-cells express MHC class I molecules and high levels of the IL-1β receptor (IL-1βR) and produce a number of chemokines, such as CCL2, which recruits macrophages producing IL-1β, TNFα and reactive oxygen species (ROS) (Figure A), all involved in apoptosis of beta cells (Figure B). Macrophages also produce IL-12 involved in the differentiation of CD8+ cytotoxic lymphocytes (CTL). CTL produce IFNγ (Figure B), which up-regulates MHC class I molecules on beta-cells and causes them to produce CXCL10, enhancing the recruitment of macrophages and also T cells (Figure C), ultimately increasing apoptosis.

1.12.1 The NOD mouse

The NOD mouse strain is the most studied animal model utilised in T1D research. The NOD mouse spontaneously develops T1D around twenty-two weeks of age (Makino *et al.*, 1980, Pop *et al.*, 2005) with pancreatic insulitis occurring around 5 weeks of age (Makino *et al.*, 1980). There is a strong gender bias in the development of T1D in the NOD mouse, with 60-80% of females developing this disease compared to 10-20% of males (Makino *et al.*, 1980, Pop *et al.*, 2005). This is not seen in humans, in whom T1D affects equal numbers of both genders (Atkinson, 2012). Interestingly, there is no gender difference with regards to pancreatic insulitis (Makino *et al*., 1980).

Like humans, polymorphisms in the MHC confer the greatest level of susceptibility (Atkinson and Leiter, 1999, Todd, 2010, Erlich *et al.*, 2008) (see Section 1.13). Other similarities include the predisposition associated with the IL-2, PTPN8 (murine PTPN22 analogue) and CTLA-4 genes (Yamanouchi *et al.*, 2007, Lowe *et al.*, 2007, Wicker *et al.*, 2005). As always, caution needs to be exercised when translating therapies from inbred animal models to the outbred human population.

1.12.2 The BB rat

Another commonly used model is the spontaneously diabetic biobreeding Wistar rat (BB rat) strain (Nakhooda *et al.*, 1978). A substantial proportion of BB rat colonies develop overt T1D between seven- to seventeen-weeks of

age (Logothetopoulos *et al.*, 1984, Nakhooda *et al.*, 1978). Unlike humans (Daneman, 2006), this is characterised by a particularly short prodrome, with destruction of the beta-cells and hyperglycaemia first evidenced a matter of days before onset (Logothetopoulos *et al.*, 1984, Nakhooda *et al.*, 1978). Compared to NOD mice (Makino *et al.*, 1980) the BB rat shows no gender bias in the development of T1D (Wallis *et al.*, 2009) and like humans, polymorphisms in the genes encoding the MHC, insulin and PTPN22 predispose to this disease (Wallis *et al.*, 2009).

1.13 The cause of T1D: the influence of genes versus environment

T1D is thought to arise from a combination of genetic susceptibility and exposure to a certain number of, as yet unknown, environmental triggers (Knip *et al.*, 2005). Genome-wide association studies (GWAS) have identified over fifty genetic polymorphisms associated with T1D susceptibility (Todd, 2010) (Figure 1.4). Many of these polymorphisms function within the immune system (Figure 1.5) (Eisenbarth, 2009) particularly MHC class I and class II genes. The genes most associated with T1D and suggested environmental triggers are discussed below.

1.13.1 MHC

Around 50% of the genetic susceptibility to T1D is conferred by the human leukocyte antigen (HLA) loci alone (Daneman, 2006), with the HLA class II genes having the most significant associations with this disease (Eisenbarth,

2009, Daneman, 2006). There are three HLA class II gene loci: DP, DQ and DR and each of these loci have genes coding for either the alpha or the beta polypeptides, which constitute the MHC class II molecules (Abbas *et al.*, 2012). The strong linkage disequilibrium present at these loci has impeded studies into the independent causative effects of each loci (Pociot and McDermott, 2002). However, it is known that several DQ alleles predispose to this disease, with around 30% of diabetics being heterozygous for the HLA-DQ2/DQ8 diplotype (HLA-DQA1*0501-DQB1*0201/DQA1*0301-DQB1*0302) (Thompson *et al.*, 1988, Pociot and McDermott, 2002). Indeed, this haplotype has been associated with a higher risk of T1D-concordance in monozygotic twins (Redondo *et al.*, 1999) and a younger age at onset in first-degree relatives (FDR) (Pugliese *et al.*, 1995). Whilst other DQ alleles also confer susceptibility, some such as HLA-DQA1*0102-DQB1*0602 (commonly referred to as DQ6) protect against the disease (Pugliese *et al.*, 1995).

A number of DR alleles are also implicated in T1D susceptibility. The gene encoding the alpha polypeptide of the HLA-DR molecules is monomorphic, therefore only the polymorphic genes encoding the beta polypeptide are examined (She, 1996). Individuals with haplotypes consisting of one HLA-DRB1*03 allele and one HLA-DRB1*04 (DR3/DR4) are at a much higher risk of developing T1D compared to those homozygous for either HLA-DRB1*03 or HLA-DRB1*04 alleles (Erlich *et al.*, 2008). Those with the diplotype: DRB1*0301-DQA1*0501-DQB1*0201/DRB1*0405-DQA1*0301-

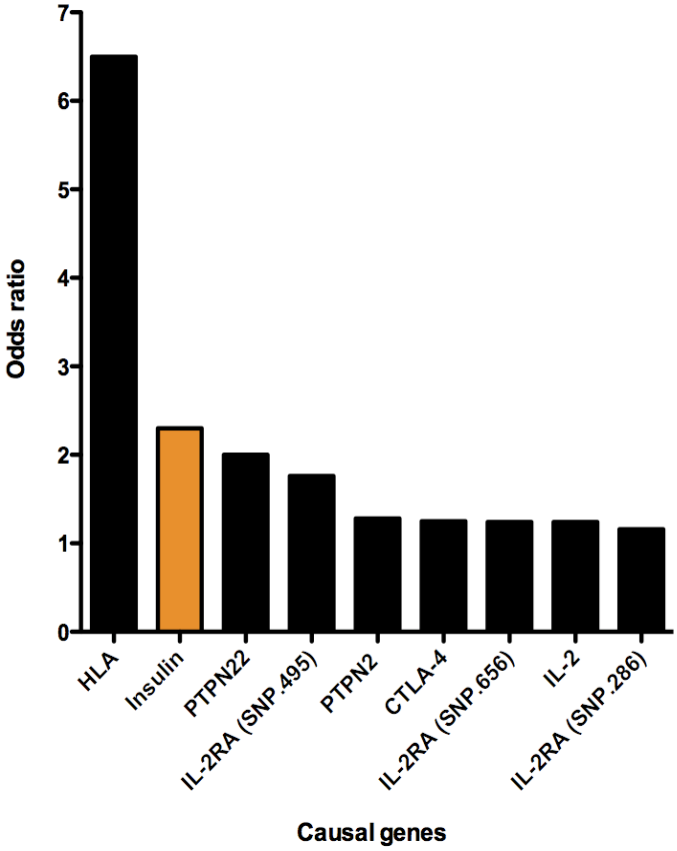

Figure 1.4 T1D-associated genetic polymorphisms

The graph shows the odds ratio of the genetic polymorphisms associated with T1D, which are discussed in this section (with the exception of PTPN2). These genes are among those conferring the greatest susceptibility to T1D. An odds ratio of 1.0 indicates no risk, values above this indicate risk and values below this indicate a protective effect. The three single-nucleotide polymorphisms (SNPs) are shown for each of the IL-2RA (CD25) genes (see Section 1.13.4 below). Bars in black show genes which function within the immune system. Data reproduced from Eisenbarth, 2009, Maier et al., 2009 and Howson et al., 2012).

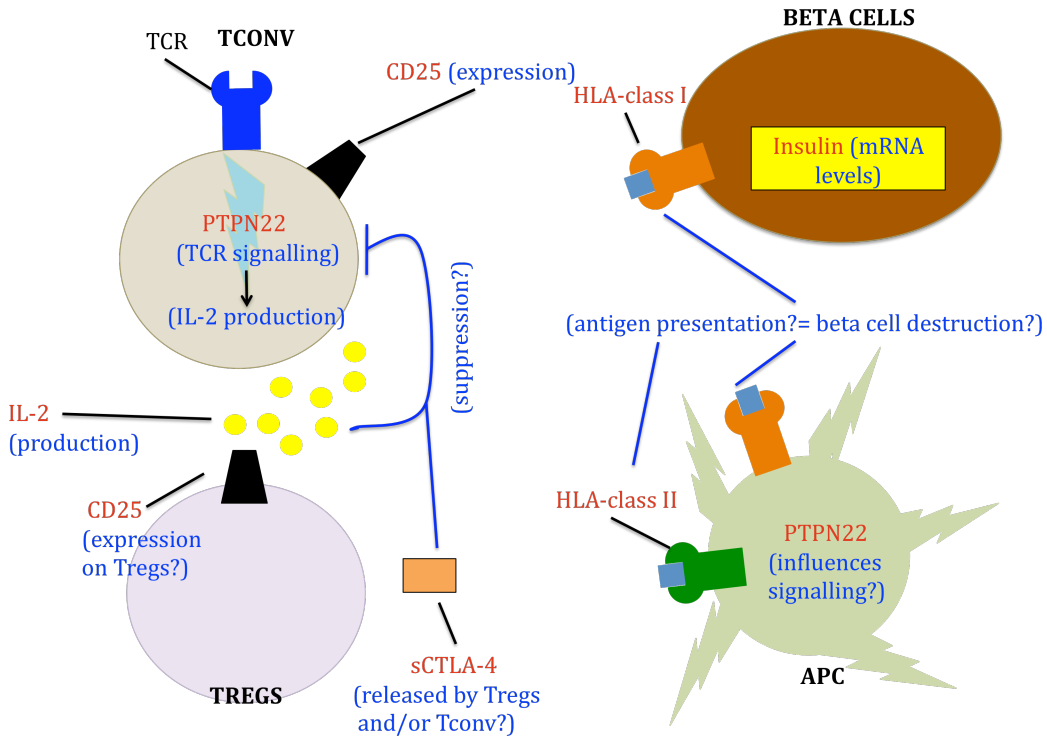

Figure 1.5 Interplay between cells regarding the products of T1D-associated genetic polymorphisms

A number of T1D-associated genetic polymorphisms (shown in red) may be involved in beta-cell destruction. For example, HLA class I and class II polymorphisms may influence antigen presentation and be implicated in beta-cell loss. The level of expression of insulin by beta-cells may also affect apoptosis. A PTPN22 polymorphism is associated with the level of T cell signalling and IL-2 production, which may influence negative selection of islet-specific T cells and also Treg maintenance and function. This polymorphism is also expressed by APC, but the phenotype it is associated with in these cells is not yet known. IL-2RA polymorphisms are associated with CD25 expression levels on T cells and possibly Tregs, which will determine Treg function, as may a polymorphism associated with levels of sCTLA-4, for example.

DQB1*0302, are particularly at risk (Erlich *et al.*, 2008). Also, a study of monozygotic twins found those who were concordant were significantly more likely to be DR3/DR4 heterozygous than homozygous for these haplotypes (Johnston *et al.*, 1983). Other haplotypes, such as DRB1*0403-DQA1*0301-DQB1*0302, have a protective effect (Erlich *et al.*, 2008). HLA-DPB1 alleles have also been shown to influence susceptibility to T1D, albeit weakly compared to DQ and DR (Noble *et al.*, 2000).

There are three HLA class I loci HLA-A, HLA-B and HLA-C (Abbas *et al.*, 2012). Of these, HLA-A*24 and HLA-B*39 appear to be the most predisposing of the HLA class I alleles (Nejentsev *et al.*, 2007). Interestingly, both of these are associated with a significantly lower age at onset. HLA-B*18 is also associated with susceptibility, whilst HLA-A*01, HLA-A*11, HLA-A*31 and HLA-B*27 are protective.

It appears that the strong influence of HLA genes in T1D susceptibility is due to differences in the binding of peptides by the MHC molecules. Although exactly how this is associated with T1D is unclear, one study demonstrated the highly susceptible DQB1*0302 allele codes for an alanine residue (neutral charge) at position 57 of the MHC molecule beta-polypeptide, whilst several protective alleles code for an aspartic acid residue (negatively charged) at this position (Kwok *et al.*, 1996). Thus, the different charges of these amino acids will affect the peptides they bind. However, a study of T1D in the Japanese population found no difference in the expression of

DQB1*0302 between diabetics and non-diabetics thus eliminating a role for this amino acid change in the disease in this cohort (Pociot and McDermott, 2002, Awata *et al.*, 1990). Further research is warranted to understand the complexity of these loci in T1D susceptibility.

1.13.2 Insulin VNTR

The second highest level of susceptibility was mapped to a variable number of tandem repeats (VNTR), which is a region of a repeated sequence of base pairs located upstream of the insulin gene on chromosome 11p15 (Vafiadis *et al.*, 1997). Three different classes of VNTR alleles are seen; two of these are important in T1D (Vafiadis *et al.*, 1997). Class I alleles have the shortest VNTR and predispose to T1D, whilst Class III have longer VNTR and protect against T1D (Vafiadis *et al.*, 1997). Class III VNTR expression by thymi from foetuses and children showed a two- to three-fold higher level of insulin mRNA compared to Class I VNTR (Vafiadis *et al.*, 1997, Pugliese *et al.*, 1997). However the opposite was seen in both foetal and adult pancreases with higher levels of insulin mRNA associated with Class I than Class III VNTR (Bennett *et al.*, 1996, Vafiadis *et al.*, 1996). Together these data suggest a scenario whereby the higher expression of insulin in the thymus leads to more productive deletion of insulin-reactive Tconv by negative selection (see Section 1.1) whilst in the periphery the lower expression of insulin would also protect against beta-cell autoimmunity. Using a human thymic epithelial cell line and a hamster beta-cell line artificially over-expressing

AIRE, both AIRE and the insulin basal promoter were shown to be essential for the production of insulin by these cells (Cai *et al.*, 2011). Furthermore, AIRE was shown to be capable of directly binding to the VNTR and the authors speculate that the longer sequence characterised by Class III VNTR may form a more stable secondary structure, enabling higher levels of insulin to be produced.

1.13.3 PTPN22

A SNP in the PTPN22 gene located on chromosome 1p13 is strongly associated with T1D (Bottini *et al.*, 2004, Smyth *et al.*, 2004, Ladner *et al.*, 2005, Onengut-Gumuscu *et al.*, 2004). This polymorphism is also associated with Graves' disease (Smyth *et al.*, 2004), juvenile arthritis (JA) (Hinks *et al.*, 2005), SLE (Kyogoku *et al.*, 2004) and RA (Begovich *et al.*, 2004), highlighting its importance in autoimmunity. *PTPN22* codes for the lymphoid-specific phosphatase (LYP), which is a suppressor of T-cell activation (Bottini *et al.*, 2006). LYP dephosphorylates the TCR-associated kinases Lck, Fyn and ZAP-70 and has been thought to do so by first forming a complex with Csk tyrosine kinase (Bottini *et al.*, 2006). The T1D-associated SNP, rs2476601, results in the substitution of arginine for tryptophan at codon 620 (Bottini *et al.*, 2004) which produces a LYP protein that cannot bind Csk (Bottini *et al.*, 2004).

Originally it was speculated that the T1D-associated *PTPN22* SNP was a loss-

of function polymorphism, failing to inhibit the activation of autoreactive Tconv. However, it was later shown that this variant is in fact a gain-of-function polymorphism showing enhanced inhibition of TCR signalling and significantly lower levels of IL-2 secretion by Tconv (Vang *et al.*, 2005). This has led others to speculate that the binding of Csk reduces rather than enables the activity of LYP and the enhanced suppression of TCR signalling may aid autoreactive Tconv in avoiding negative selection in the thymus (see Section 1.1) (Bottini *et al.*, 2006). Also, given the importance of IL-2 in maintaining Tregs (Taams *et al.*, 2001) the reduction in IL-2 production by Tconv may also reduce their ability to maintain the Tregs, which subsequently suppress their function. It should be noted that LYP is also expressed by B cells (Bottini *et al*., 2006) and this variant is associated with both significantly lower percentages and impaired activation of memory B cells (Arechiga *et al*., 2009, Rieck *et al*., 2007). It is thought that this reduction in B cell receptor signalling also allows the escape of autoreactive B cells from the bone marrow (Rieck *et al*., 2007).

1.13.4 CD25

A number of SNPs in the IL-2RA gene on chromosome 10p15.1 have been associated with T1D (Lowe *et al.*, 2007, Maier *et al.*, 2009). SNP rs11594656 (SNP.656) is located in non-coding 5' region outside of the IL-2RA gene whilst SNP rs12722495 (SNP.495) and SNP rs2104286 (SNP.286) are situated in non-coding regions of intron 1 (Dendrou *et al.*, 2009). SNP.286 is

also associated with multiple sclerosis (MS) (Maier *et al.*, 2009) and JA (Hinks *et al.*, 2009). Further investigation using these three SNPs revealed the presence of four common *IL-2RA* haplotypes, which are associated with autoimmunity (Dendrou *et al.*, 2009) (Figure 1.6). One of these haplotypes, the Fully Susceptible haplotype, where the susceptible allele is present at all three SNPs, predisposes to autoimmunity and is present in 54% of T1D and 48% of non-diabetics (Dendrou *et al.*, 2009). The three other haplotypes all have a protective effect in comparison to the Fully Susceptible haplotype. The Protective P1 haplotype differs from the Fully Susceptible haplotype by the presence of protective alleles at SNP.495 and SNP.286. A genotype-phenotype study found this haplotype is associated (due to the protective allele at SNP.495) with a higher expression of CD25 on memory CD4+ Tconv and higher levels of IL-2 production by these cells upon activation (Dendrou *et al.*, 2009). The presence of a protective allele at SNP.286 also means this haplotype is associated with lower percentages of CD25+ naïve CD4+ Tconv.

The Protective P2 haplotype has a protective allele at SNP.656 and is associated with an increase in CD25 expression by CD14+ CD16+ monocytes (Dendrou *et al.*, 2009). The Protective P3 haplotype has a protective allele at SNP.286 and like the Protective P1 haplotype is associated with lower percentages of CD25+ naïve CD4+ Tconv (Dendrou *et al.*, 2009). However, the study of the haplotypes revealed that unlike the Protective P1 and Protective P2 haplotypes, the Protective P3 haplotype was not found to be associated with T1D, occurring in around 15% of both diabetics and non-

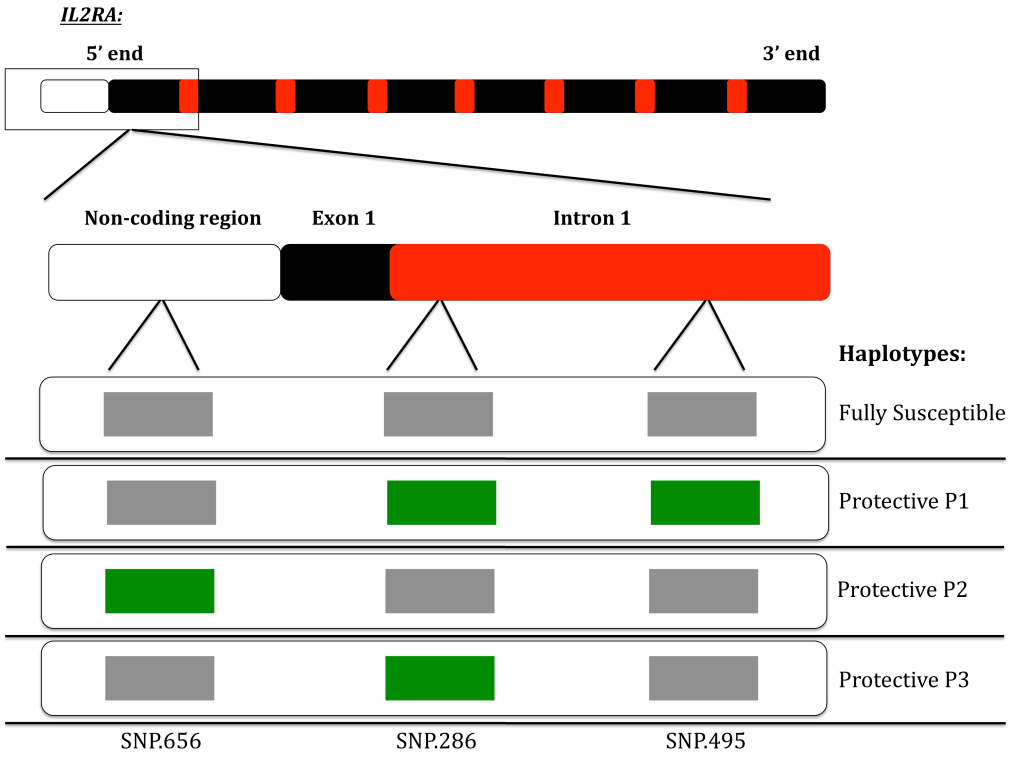

Figure 1.6 The four common autoimmune-associated IL-2RA haplotypes can be defined by the alleles at three IL-2RA SNPs *Four common autoimmune-associated IL-2RA haplotypes are present on chromosome 10. Each of these haplotypes is defined by the presence or absence of protective alleles at three SNPs in the non-coding region of the IL-2RA gene: SNP.656, which resides in the non-coding region and SNP.286 and SNP.495, which reside in intron 1. The Fully Susceptible haplotype has the susceptible allele at each SNP, whilst the Protective P1 haplotype has protective alleles at SNP.495 and SNP.286. The Protective P2 haplotype has a protective allele at SNP.286 and the Protective P3 haplotype has a protective allele at SNP.286. Green rectangles represent protective alleles whilst grey rectangles represent susceptible alleles. Black boxes represent exons and red boxes represent introns. This figure is based upon that from Dendrou et al. (2009).*

diabetics (Dendrou *et al.*, 2009). Therefore, it is not clear whether SNP.286 influences T1D predisposition, or if this association is due to its high linkage disequilibrium with SNP.495. This remains unknown as a haplotype with the protective allele at just SNP.495 and not SNP.286 has not been reported and therefore cannot be investigated.

1.13.5 CTLA-4

A SNP, rs3087243, in the CTLA4 gene located on chromosome 2q33 has been associated with T1D and causes an alanine to threonine substitution at position 49 (Chen *et al.*, 2013, Nistico *et al.*, 1996, Ueda *et al.*, 2003). It is also associated with Graves' disease and autoimmune hypothyroidism (Ueda *et al.*, 2003). Although it appears that genotype-phenotype studies are lacking, this T1D-susceptible polymorphism has been associated with lower levels of soluble CTLA-4 (sCTLA-4) relative to the full-length protein (Ueda *et al.*, 2003) and lower levels of sCTLA-4 have been reported in the sera of ROT1D compared to non-diabetics (Ryden *et al.*, 2012). The exact role of sCTLA-4 in immune regulation and how distinct its function is from cell-surface CTLA-4 is still debatable. Tregs from transgenic NOD mice in which sCTLA-4 was silenced by RNA interference showed impaired suppressive function compared to wild type mice, despite the fact these cells still expressed cell surface CTLA-4 (Gerold *et al.*, 2011). This suggests the roles of sCTLA-4 and cell-surface CTLA-4 are not identical.

1.13.6 Environmental factors

Despite the strong association of the HLA loci with T1D (Todd, 2010) the majority of individuals with HLA susceptibility never develop this disease (Knip *et al.*, 2005). Also studies of T1D in monozygotic twins have found concordance never reaches 100% (Kaprio *et al.*, 1992, Olmos *et al.*, 1988, Redondo *et al.*, 2001, Barnett *et al.*, 1981) (see Chapter Six, Section 6.1.1). This implicates the involvement of environmental factors in the aetiology of this disease, especially as the striking increase in T1D worldwide cannot be explained by genetic susceptibility alone (Knip *et al.*, 2005).

Several studies have implicated viral infections in triggering T1D, particularly enteroviruses (Yeung *et al.*, 2011, Smith *et al.*, 1998) (although others have found no such link (Graves *et al.*, 2003)) and also morbilliviruses (Lipman *et al.*, 2002) and rotaviruses (Honeyman *et al.*, 1998). Viral infections have been suggested to cause beta-cell destruction by directly damaging these cells or indirectly via molecular mimicry (von Herrath *et al.*, 2003). However, the difficulty in associating viruses with T1D onset comes from the inability to determine the exact point of exposure in the majority of patients due to the long and highly variable prodrome seen in T1D. In addition, the notion that viruses trigger T1D is at odds with the hygiene hypothesis (discussed below) (Okada *et al.*, 2010). Although a T1D-associated SNP in the interferon-induced helicase (IFIH1) gene has been identified, which encodes a pathogen recognition receptor for viral double-stranded RNA (Smyth *et al.*, 2006). Although the phenotype of this SNP is

currently unknown, it may be implicated in this proposed link between T1D and viruses.

The incidence of T1D varies greatly on a global scale, with the Nordic countries, particularly Finland, having some of the highest rates recorded (Karvonen *et al.*, 2000). Due to these geographical differences, it has been suggested that a lack of vitamin D is associated with T1D. The enzyme CYP27B1, which produces the active form of vitamin D is expressed by a number of leukocytes including activated T cells, B cells, macrophages and dendritic cells, thus showing an immunological function for this vitamin (Bikle, 2009). In support of a role in T1D a SNP present in the CYP27B1 gene is associated with this disease (Bailey *et al.*, 2007). Certain dietary factors have also been investigated. Early exposure to cow's milk has been associated with T1D (Hypponen *et al.*, 1999, Cavallo *et al.*, 1996) and has been shown to induce a cellular and humoural response to beta-casein (Cavallo *et al.*, 1996) and a humoural response to bovine insulin (Vaarala *et al.*, 1999). The early introduction to cow's milk has also been associated with the appearance of autoantibodies in children with the T1D-susceptible PTPN22 polymorphism, suggesting the presence of interplay between genetic and environmental factors (Lempainen *et al.*, 2009). Other environmental factors suggested include the early introduction of gluten, especially in children with HLA susceptibility (Ziegler *et al.*, 2003); weight gain; obesity and increase in height during infancy (Hypponen *et al.*, 1999, Hypponen *et al.*, 2000). These findings lend support to the 'overload

hypothesis', which states that the increase in weight and height, coupled with an increase in viral infections and cold climates, places a stress on beta-cells which makes them susceptible to apoptosis (Dahlquist, 2006).

It has been suggested that the microbiome is implicated in allergy and autoimmunity (Giongo *et al.*, 2011, Kalliomaki *et al.*, 2001, Lathrop *et al.*, 2011). In humans, delivery by caesarean section has been associated with T1D (Cardwell *et al.*, 2008) and another study has shown that the mode of delivery is linked with the proportions of different bacterial strains on the skin of newborn babies (Dominguez-Bello *et al.*, 2010). The mode of delivery at birth may also affect gut bacteria (Atkinson and Chervonsky, 2012), thought to be important in T1D development as well. A prospective study of diabetic children revealed that those who later developed T1D had a less stable and less diverse gut microbiota compared to controls (Giongo *et al.*, 2011) and similar findings have been reported in children with allergies (Kalliomaki *et al.*, 2001).

These data could be deemed to lend support to the 'hygiene hypothesis', which states that the improvement in hygiene and sanitation and subsequent decrease in infection in westernised countries has resulted in inappropriate immune responses to self- and harmless foreign antigens, leading to allergies and autoimmunity (Okada *et al.*, 2010). How this is caused is unclear, however it has been suggested that it arises from a deviation from a T_H1 to a T_H2 bias. Although this may explain allergies,

autoimmune diseases like T1D are thought to involve mainly T_H1 responses (Lehuen *et al.*, 2010). Perhaps the most applicable theory is the 'threshold hypothesis', which tries to reconcile the involvement of genetic and environmental factors in causing this disease (Wasserfall *et al.*, 2011). This hypothesis suggests that when all factors are taken into account, with nearly all (except HLA) contributing a small level of predisposition, a threshold can be created above which T1D is imminent but below which the risk of T1D is less predictable (Wasserfall *et al.*, 2011).

1.14 The role of Tregs in T1D

1.14.1 Treg frequency in peripheral blood

The majority of studies examining Treg frequency and function in humans with T1D have been performed in the peripheral blood. Most reports show no difference in Treg frequency (Liu *et al.*, 2006b, Lindley *et al.*, 2005, Lawson *et al.*, 2008, Brusko *et al.*, 2005, Putnam *et al.*, 2005, Ferraro *et al.*, 2011) although one study identified reduced numbers of Tregs in both ROT1D and LST1D patients compared to controls (Kukreja *et al.*, 2002). However, others have suggested that this may be due to recruitment of control individuals who were significantly older than the ROT1D cohort (Tree *et al.*, 2006). Whilst the gating strategy used to select the Treg population was not reported, it would appear that they selected CD4+ CD25+ T cells; the frequency of which has been demonstrated to increase with age (Brusko *et al.*, 2005). Although the reason why LST1D donors also

showed significantly lower numbers of Tregs is unclear, it may be due to the low numbers of these patients studied (n=9) or the gating strategy used.

1.14.2 Peripheral blood Treg function

When Treg function in T1D was examined, a defect in the suppression of Tconv proliferation *in vitro* was identified by several studies (Lindley *et al.*, 2005, Lawson *et al.*, 2008, Brusko *et al.*, 2005, Jana *et al.*, 2010, Glisic-Milosavljevic *et al.*, 2007b). This defect was seen in patients with both ROT1D (Lindley *et al.*, 2005, Jana *et al.*, 2010, Glisic-Milosavljevic *et al.*, 2007b) and LST1D (Lawson *et al.*, 2008, Glisic-Milosavljevic *et al.*, 2007b) suggesting that it is evident throughout the course of disease. Differences in suppression can also be seen at the pre-diabetic stage, with those at risk of developing the disease exhibiting impaired suppression compared to controls, yet at a higher level than that of ROT1D (Jana *et al.*, 2010). The reasons for this intermediary level of suppression in pre-diabetics are unclear, but studies in NOD mice have reported the progressive decline of Treg function in animals as they age (Pop *et al.*, 2005, You *et al.*, 2005).

In ROT1D, significantly reduced suppression of IFNγ production and a markedly higher IFNγ:IL-10 ratio in Tconv-Treg co-cultures was reported compared to control donors (Lindley *et al.*, 2005). Tregs from ROT1D subjects also had significantly more CD69 and intracellular CTLA-4 than controls, suggestive of an activated phenotype. Interestingly, despite the

defect in suppression of Tconv proliferation in LST1D patients, no defect in IFNγ suppression was seen (Lawson *et al.*, 2008). The reasons for this are unclear, but may be due to the presence of a pro-inflammatory milieu in ROT1D, in whom beta-cells are actively being destroyed, whereas this process has largely subsided in LST1D. Crossover suppression assays (see Section 1.8.4) revealed an overall increased resistance of Tconv from diabetics to Treg suppression (Lawson *et al.*, 2008, Schneider *et al.*, 2008). However, in the minority of T1D subjects tested, a functional defect in the Tregs themselves was also seen (Lawson *et al.*, 2008). This differs from several other autoimmune diseases, in which the Treg population showed a lower suppressive function (Kriegel *et al.*, 2004, Viglietta *et al.*, 2004, Valencia *et al.*, 2007) (see Section 1.8.4).

1.14.3 Tregs from affected tissues

As previously stated, most studies in humans only examine Tregs from the peripheral blood. However, one study was able to acquire pancreatic lymph nodes (PLNs) from LST1D undergoing pancreatic transplantation (Ferraro *et al.*, 2011). As a comparison, PLNs were harvested from brain-dead control donors (although cytokine storms, which occur upon brain death may have led to possible confounding factors (Skrabal et al., 2005)). Similar to analysis of peripheral blood, there was no difference in the frequency of Tregs in the PLNs of LST1D and controls. The authors were limited to only six LST1D donors for functional analysis of PLN Tregs, but these did show

significantly lower levels of suppression compared to control Tregs. When crossover Treg suppression assays were used (as described earlier in Section 1.8.4) this defect appeared to be intrinsic to the Tregs themselves.

1.14.4 Tregs from NOD mice

All other reports of Tregs at tissue-specific sites come from studies of the NOD mouse model. However, many of these results are contradictory. Some studies have shown no difference in the number of Tregs in the NOD thymus, spleen or PLN as mice age, prior to development of T1D (Tang *et al.*, 2008), while others show a gradual decrease in Treg number in the lymph nodes (LNs) and the spleen (Alard *et al.*, 2006, Wu *et al.*, 2002). At onset, one study found the percentage of Tregs in the PLN to increase (Tang *et al.*, 2008), whilst another study reported that despite an increase in Treg number, the parallel rise of Tconv resulted in an overall decrease in Treg percentage (Pop *et al.*, 2005). Also, there was an overall decrease in the percentage of Tregs in the islets at onset, due to the concomitant infiltration of Tconv (Pop *et al.*, 2005, Tang *et al.*, 2008). These intra-islet Tregs at onset displayed lower levels of CD25 and Bcl-2, suggesting these cells were gradually lost by apoptosis (Tang *et al.*, 2008). This was further supported by findings demonstrating CD4+ T cells in the islets produced significantly lower levels of IL-2 mRNA (Tang *et al.*, 2008), a cytokine which rescues Tregs from apoptosis (Taams *et al.*, 2001). Also, others have reported a decrease in FoxP3 expression, a transcription factor maintained by IL-2

(Zorn *et al.*, 2006) in diabetic NOD Tregs from lymphoid tissues (Manirarora *et al.*, 2008, Pop *et al.*, 2005).

Several studies in the NOD mouse have also found Treg function to be defective (Alard *et al.*, 2006, D'Alise *et al.*, 2008, Wu *et al.*, 2011, You *et al.*, 2005, Pop *et al.*, 2005) although the population(s) responsible is debatable. Some groups have identified the NOD Tconv population to show higher resistance to Treg function (D'Alise *et al.*, 2008, Wu *et al.*, 2011, Pop *et al.*, 2005, You *et al.*, 2005) with two of these studies demonstrating a marginal reduction in Treg function also (You *et al.*, 2005, Pop *et al.*, 2005) comparable with the human studies (Lawson *et al.*, 2008, Schneider *et al.*, 2008). The reason for this increased Tconv refraction to Treg suppression is unknown, but D'Alise *et al.* (2008) attributed it to a genetic cause as it was also observed in a variant of the NOD mouse, protected from insulitis. Two other studies reported a gradual decline in Tconv sensitivity to Treg function as NOD mice age (Pop *et al.*, 2005, You *et al.*, 2005), which is thought to be related to the decline in Treg expression of TGFβ (Pop *et al.*, 2005) together with an age-related Tconv-resistance to TGFβ (You *et al.*, 2005). Interestingly, the percentage of Tregs co-expressing TGFβ and FoxP3 decreased with age, but only in female mice, which are at a much higher risk of developing T1D than males (Pop *et al.*, 2005).

One group, however, has reported APC from NOD mice to be responsible for

the reduction in Treg suppression as they are poor stimulators of Tregs (Alard *et al.*, 2006). Significantly lower levels of suppression were seen when APC from NOD mice were added to NOD Tconv-Treg *in vitro* suppression assays compared to C57BL/6 mouse (B6) APC. When APC from NOD mice over thirty weeks of age (and therefore unlikely to develop T1D) were added to suppression assays, similar levels of Treg suppression to B6 APC were produced, suggesting that these NOD mice are protected because their APC can sufficiently activate the Tregs.

1.14.5 Defective IL-2 production and signalling in T1D

A defect in IL-2 production has been identified in both the NOD mouse and in individuals with T1D (Yamanouchi *et al.*, 2007, Zier *et al.*, 1984, Roncarolo *et al.*, 1988, Kaye *et al.*, 1986). To assess how production of IL-2 in NOD mice compared with B6 mice, which do not develop T1D, Todd and colleagues examined the T1D-associated loci, insulin-dependent diabetes susceptibility 3 locus (*Idd3*), which includes the IL-2 gene. A congenic strain of the NOD mouse expressing a 650kb region of *Idd3* from B6 mice (NOD.B6 *Idd3*), which included *IL-2*, was generated (Yamanouchi *et al.*, 2007). NOD.B6 *Idd3* mouse thymocytes and splenocytes produced two-fold higher levels of IL-2 compared to NOD mice, a significant difference (Yamanouchi *et al.*, 2007). The NOD.B6 *Idd3* mouse is resistant to T1D development and further investigation revealed that these mice had higher percentages of Tregs in the mesenteric LNs and PLNs, which suppressed Tconv to a

significantly higher degree both *in vitro* and *in vivo*. Furthermore, administration of low-doses of IL-2 to NOD mice can prevent (Tang *et al.*, 2008) and even reverse T1D; possibly implicating the increased percentages of Tregs seen as a result of this therapy (Grinberg-Bleyer *et al.*, 2010).

In humans, T cells from diabetics produce significantly lower levels of IL-2 compared to non-diabetics (Roncarolo *et al.*, 1988, Zier *et al.*, 1984, Kaye *et al.*, 1986, Giordano *et al.*, 1989), which is evident in both ROT1D (Roncarolo *et al.*, 1988, Giordano *et al.*, 1989) and LST1D (Zier *et al.*, 1984, Giordano *et al.*, 1989) and is independent of insulin therapy (Roncarolo *et al.*, 1988). A study in monozygotic twins discordant for T1D showed a positive correlation in the level of IL-2 produced by both twins of each pair (Kaye *et al.*, 1986). However despite this, the diabetic twins produced significantly lower levels of IL-2, suggesting that although under some degree of genetic control, the defect in IL-2 is closely linked to T1D, although the exact mechanism involved is not clear.

An increase in Treg apoptosis was seen in ROT1D (Glisic-Milosavljevic *et al.*, 2007a, Glisic-Milosavljevic *et al.*, 2007b) and high-risk non-diabetics (Glisic-Milosavljevic *et al.*, 2007b), which correlates with data from the NOD mouse at onset (Tang *et al.*, 2008). Analysis from ROT1D demonstrated an increase in the expression of pro-apoptotic genes in their Tregs, resembling that of Tregs from healthy controls when subjected to IL-2 deprivation (Jailwala *et al.*, 2009). It is perhaps surprising that Treg apoptosis is not significantly

higher in LST1D than controls (Glisic-Milosavljevic *et al.*, 2007a, Glisic-Milosavljevic *et al.*, 2007b) as IL-2 production is also decreased in these individuals (Zier *et al.*, 1984, Giordano *et al.*, 1989). This suggests that the pro-inflammatory milieu present in ROT1D may exacerbate these apoptotic conditions.

A defect in the IL-2 signalling cascade is also seen in T1D (Long *et al.*, 2010, Long *et al.*, 2011). Even in the presence of exogenous IL-2 *in vitro* this defect was still evident, as Tregs from diabetics had significantly poorer maintenance of FOXP3 compared to controls when cultured for ninety-six hours with IL-2 (Long *et al.*, 2010). Diabetic Tregs showed significantly less phosphorylation of STAT5a also, thought to be due to a possible defect in CD122-mediated signalling, as this same result was found with IL-15 but not IL-7 stimulation, the latter of which does not signal through CD122. Further analysis also revealed that this diabetic cohort expressed higher levels of PTPN2, a negative regulator of the IL-2 signalling pathway, compared to controls (Long *et al.*, 2010). A later genotype-immunophenotype study examining a T1D-associated *PTPN2* haplotype in non-diabetics was conducted by this group (Long *et al.*, 2011). Unexpectedly, the non-diabetic donors with the susceptible haplotype expressed lower levels of PTPN2 but also showed lower levels of STAT5 phosphorylation (Long *et al.*, 2011). This indicates that an unknown compensatory mechanism is involved.

1.15 Introduction to the work in this thesis

T1D appears to arise from a combination of genetic susceptibility (Todd, 2010) and exposure to certain environmental triggers (Knip *et al.*, 2005). Defective Treg function is seen throughout the disease (Lindley *et al.*, 2005, Jana *et al.*, 2010, Lawson *et al.*, 2008) demonstrating that it is a stable phenotype. The overall hypothesis of this thesis is that the defect in Treg function in T1D is genetically-determined and not a consequence of the disease itself. GWAS studies have identified over fifty genetic polymorphisms associated with T1D (Todd, 2010) the majority of which function within the immune system and the first two studies in this thesis examined the effect two *IL-2RA* SNPs, SNP.286, (Chapter Four) and SNP.495 (Chapter Five) had on the ability of Tregs to suppress different subpopulations of Tconv. The allele at SNP.286 is associated with the percentage of CD25+ CD4+ naïve Tconv and it was hypothesised that the susceptible allele, which is associated with higher percentages of CD25+ naïve Tconv, reduced these cells ability to maintain Tregs, ultimately reducing suppression. Likewise, the allele at SNP.495 is associated with the level of CD25 on memory Tconv and again it was hypothesised that the susceptible allele, which is associated with lower levels of CD25 on memory Tconv, reduced these cells ability to maintain Tregs, ultimately reducing suppression. The aims of each of these chapters were to: assess whether these SNPs also influenced CD25 expression by Tregs; examine Treg fitness; examine Treg suppression of Tconv proliferation and examine Tconv resistance to Treg suppression.

The third study tested the hypothesis that Treg function is genetically-determined, by examining T1D-discordant monozygotic twins and age- and gender-matched healthy controls (Chapter Six). This chapter aimed to examine Treg suppression of Tconv proliferation and Tconv resistance to Treg function. Finally, the IL-2 signalling cascade is known to be defective in T1D (Long *et al.*, 2010, Long *et al.*, 2011) and may have implications on Treg function as these cells require this cytokine for maintenance and function (Taams *et al.*, 2001, Zorn *et al.*, 2006). Chapter Seven tested the hypothesis that this defective cascade was genetically-determined by using in the same cohort of monozygotic twins and controls as Chapter Six. See Chapter Three, Table 3.2 for a detailed summary of these studies.

Chapter Two: Experimental procedures

2.1 Equipment

Autoclave (PriorClave Ltd.)

Cell irradiator (TCell-1000S) (MDS Nordion)

Centrifuge (Model CR22, Jouan)

Centrifuge (Sorvall Legend RT) (Thermo Scientific)

Centrifuge (ScanSpeed 1236) (Lab Mode)

CoolCell® (BioCision)

FACSAria II (Becton Dickinson)

Filtermate harvester 196 (Packard)

Flow cytometer (3-laser Canto II) (Becton Dickinson)

Haemocytometer (Neubauer)

Liquid nitrogen storage tank (Biorack 3000) (Statebourne)

Microplate spectrophotometer (Bio-Rad)

Microbeta Trilux Beta Counter (Wallac)

Microscope (Nikon)

Microwave (Hinari)

pH meter 3310 (Jenway)

Plate shaker (Stuart® Gyro-rocker SSL3) (Bibby Scientific Ltd.)

Plate shaker (Nunc-Immuno™ Wash 12) (Nunc™)

Refridgerator (Lec)

Stirling Mixer Roller (Sandrest Ltd.)

Tissue culture incubator (CO_2 regulated) (Thermo Electron Corporation)

Vortex (IKA)

Water bath (Sub Aqua 12 Plus) (Grant)

-80° freezer (Thermo Electron Corporation)

2.2 Plastics & laboratory consumables

Blood collection tubes coated with 170 IU of sodium heparin (BD Vacutainer, GP Supplies)

Centrifuge tubes (15ml, 50ml) (Corning Incorporated)

Cryovials (Appleton Woods)

Fibreglass filter mats (Wallac)

Pastettes (Alpha Laboratories)

Pasteur pipettes (Appleton Woods)

Petri dishes 35mm and 100mm (Corning Incorporated)

Plastic sample bags (Wallac)

Plate sealers (Appleton Woods)

Stripettes (5ml, 10ml 25ml) (Corning Incorporated)

Universal tubes (7ml, 30ml) (Sterilin)

0.5ml tubes (Appleton Woods)

1.5ml tubes (Appleton Woods)

5ml polypropylene non-sterile FACS tubes (Becton Dickinson)

5ml polypropylene sterile FACS tubes (Becton Dickinson)

5ml polypropylene sterile FACS tubes with filter cap (Becton Dickinson)

96-well U-bottom plates (Corning Incorporated)

96-well Flat bottom plates (Iwaki)

96 well MicroWell™ MaxiSorp™ (Sigma-Aldrich)

2.3 Chemicals & reagents

Betaplate scintillation fluid (Perkin Elmer)

BD Lyse/fix buffer (BD Biosciences)

BD Fix buffer I (BD Biosciences)

BD Phosflow™ Phosflow buffer I (BD Biosciences)

BD Phosflow™ Perm buffer III (BD Biosciences)

Bovine Serum Albumin (BSA) (Bovine Fraction V Solution 7.5%) (Sigma-Aldrich)

Dimethyl sulfoxide (DMSO) (Sigma-Aldrich)

Dulbecco's phosphate buffered saline (dPBS) (Gibco)

Dynabeads® Human T-Activator anti-CD3/anti-CD28 beads (Invitrogen)

Ethylenediaminetetraacetic acid (EDTA) 0.5M, pH8 (Fluka)

Foetal calf serum (FCS) (heat-inactivated) (PAA Labs Ltd)

FCS (heat-inactivated) (Gibco)

FOXP3 Fix/Perm Buffer (Biolegend)

FOXP3 Permeabilisation Buffer (Biolegend)

Fungizone (Amphotericin B) (Invitrogen)

4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 1M (Gibco)

Human AB serum (hAB) (heat-inactivated) (PAA Labs Ltd)

Human recombinant IL-2 (Proleukin) (Chiron Corporation)

Lymphoprep (Axis Shield PoC AS)

Methanol (AnalR)

Penicillin/Streptomycin (Invitrogen)

Phosphate buffered saline (PBS) pH7.4 (Gibco)

Roswell Park Memorial Institute medium (RPMI) 1640 media with Glutamax and 25mM HEPES (Gibco)

Sodium bicarbonate (Fluka)

Sodium carbonate (Sigma-Aldrich-Aldrich)

Sulphuric acid (BDH)

3[H]-thymidine (Perkin Elmer)

Tween-20 (U-CyTech Biosciences)

X-VIVO™ 15 media (Lonza)

YO-PRO®-1 (Invitrogen)

7-Aminoactinomycin D (7-AAD) (Invitrogen)

2.4 Solutions

2.4.1 Buffers & solutions

Coating Buffer

Distilled water with 0.10 moles of sodium bicarbonate and 0.034 moles of sodium carbonate. pH 9.5. Store at room temperature.

Collection Buffer

1x dPBS supplemented with 10% FCS (Gibco) and 25mM HEPES. Store at 4°C.

ELISA Assay Diluent

1x dPBS with 10% FCS.

ELISA Wash Buffer

1x dPBS with 0.05% Tween-20

FACS Buffer

1x dPBS supplemented with 1% FCS (Gibco) and 2mM EDTA. Store at 4°C.

Re-suspension Buffer

1x dPBS supplemented with 10% FCS (Gibco), 25mM HEPES and 2mM EDTA. Store at 4°C.

Stain Buffer

1x PBS (pH7.4) supplemented with 0.2% BSA. Store at 4°C.

2.4.2 Cell culture solutions

RPMI P/F/S

RPMI 1640 media with Glutamax and 25mM HEPES (Gibco) supplemented with 100µg/ml penicillin/streptomycin and 100µg/ml fungizone (P/S/F).

RPMI 10%

RPMI 1640 media with Glutamax and 25mM HEPES (Gibco) supplemented with with 10% hAB with 100µg/ml penicillin/streptomycin and 100µg/ml fungizone.

X-VIVO 1%

X-VIVO™ 15 media supplemented with 1% hAB with 100µg/ml penicillin/streptomycin and 100µg/ml fungizone.

X-VIVO 5%

X-VIVO™ 15 media supplemented with 5% hAB with 100µg/ml penicillin/streptomycin and 100µg/ml fungizone.

Proleukin

Proleukin (recombinant human IL-2) was dissolved in X-VIVO™ 15 media at $2x10^5$ IU/ml. Aliquots of 1ml were stored at -80°C. Thawed aliquots were stored for no longer than 10 days at 4°C. Freshly thawed aliquots were used in the pSTAT5a, FOXP3, apoptosis and Tr1 generation assays.

2.5 Antibodies

Purified Mouse monoclonal anti-CD3 (Clone Hit3a) (Becton Dickinson Biosciences)

Mouse monoclonal anti-CD4-AF700 (Clone RPA-T4) (Biolegend)

Mouse monoclonal anti-CD4-eFluor450 (Clone SK3) (eBioscience)

Mouse monoclonal anti-CD4-QDot605 (Clone S3.5) (Invitrogen)

Mouse monoclonal anti-CD4-FITC (Clone SK3) (Becton Dickinson Biosciences)

Mouse monoclonal anti-CD14-AF488 (Clone HCD14) (Biolegend)

Mouse monoclonal anti-CD14-APC-Cy7 (Clone HCD14) (Biolegend)

Mouse monoclonal anti-CD19-AF488 (Clone HIB19) (Biolegend)

Mouse monoclonal anti-CD25-APC (Clone 2A3) (Becton Dickinson Biosciences)

Mouse monoclonal anti-CD25-APC (Clone M-A251) (Becton Dickinson

Biosciences)

Mouse monoclonal anti-CD25-PE (Clone 2A3) (Becton Dickinson Biosciences)

Mouse monoclonal anti-CD25-PE (Clone M-A251) (Becton Dickinson Biosciences)

Purified Mouse monoclonal anti-CD28 (clone CD28.2) (Becton Dickinson Biosciences)

Mouse monoclonal anti-CD45RA-AF700 (clone H100) (Biolegend)

Mouse monoclonal anti-CD45RA-eFluor450 (Clone H100) (eBioscience)

Mouse monoclonal anti-CD45RA-PB (Clone L48) (Becton Dickinson Biosciences)

Purified mouse monoclonal anti-CD46 (clone E4.3) (Becton Dickinson Biosciences)

Mouse monoclonal anti-CD127-PerCP-Cy5.5 (clone eBioRDR5) (eBioscience)

Mouse monoclonal anti-FOXP3-AF647 (clone 259D) (Biolegend)

Mouse monoclonal anti-FOXP3-PE (clone 259D) (Biolegend)

Armenian hamster monoclonal anti-Helios-FITC (clone 22F6) (Biolegend)

Mouse monoclonal anti-pSTAT5a-AF488 (clone 47/Stat5 (pY694)) (Becton Dickinson Biosciences)

Mouse monoclonal anti-pSTAT5a-AF647 (clone 47/Stat5 (pY694)) (Becton Dickinson Biosciences)

Mouse monoclonal IgG1 PerCPCy5.5 isotype control (clone P3) (eBioscience)

Mouse monoclonal IgG1 PE isotype control (Clone MOPC-21) (Becton Dickinson Biosciences)

2.6 Commercial kits

BD Phosflow™ kit (BD Biosciences)

Contains Phosflow Buffer I, Perm Buffer III and Mouse monoclonal anti-pSTAT5a-AF647 (clone 47/Stat5 (pY694) (BD Biosciences)

FOXP3 Fix/Perm Buffer Set (Biolegend)

Contains Fix/Perm Buffer and Permeabilisation Buffer

Human IFNγ ELISA MAX™ Standard Sets (Biolegend)

Contains, anti-IFNγ capture antibody, anti-IFNγ detection antibody, recombinant IFNγ protein for use as standards, avidin-horseradish peroxidase (HRP)

Human IL-10 ELISA MAX™ Standard Sets (Biolegend)

Contains, anti-IL-10 capture antibody, anti-IL-10 detection antibody, recombinant IL-10 protein for use as standards, avidin-HRP

Substrate reagent pack (R&D Systems)

Contains stabilised hydrogen peroxide and stabilised tetramethylbenzidine (TMB)

2.7 Software

FACSDiva (Becton Dickinson)

FlowJo (Tree Star Inc)

GraphPad Prism (GraphPad Software Inc)

Microbeta windows (Perkin Elmer)

Microplate manager (Bio-Rad)

2.8 Experimental procedures

2.8.1 Peripheral blood samples

Following informed consent, peripheral blood samples were obtained by venipucture and placed directly into collection tubes coated with the anti-coagulant sodium heparin. The blood was diluted with an equal amount of RPMI P/S/F and was either processed straight away or placed on a roller overnight at room temperature. Blood received for the genotype-immunophenotype studies (Chapters Four and Five) and the monozygotic twin study (Chapters Six and Seven) was left overnight, ready for processing the next day.

2.8.1.1 Optimisation studies

All healthy blood donors required for the optimisation of the assays used in this thesis were recruited from the local cohort of blood donors at King's College London.

2.8.1.2 Genotype-immunophenotype studies

To examine the allele at SNP.286 (Chapter Four) healthy non-diabetic donors were recruited from either the local cohort of blood donors at King's

College London or Guy's Hospital Diabetes Clinic. A pair-wise study coupling heterozygous protected donors (having one protective allele at SNP.286 and one susceptible allele) with age- and gender-matched susceptible donors (having two susceptible alleles at SNP.286) was conducted. Eight pairs of donors were enrolled (protected donors: five males, 3 females, mean age 35.38 ± standard deviation (S.D.): ± 9.30 and susceptible donors: five males, 3 females, mean age 31.38 ± S.D.: 6.05).

The Cambridge BioResource was responsible for recruiting healthy non-diabetic donors for the Protective P1 haplotype study (Chapter Five). Ten pairs of donors were enrolled, each pair consisting of one individual homozygous for the Protective P1 haplotype and one individual homozygous for the Fully Susceptible haplotype. The immunophenotypes of the donors had been previously analysed at Cambridge University and those with the representative CD4+ memory conventional T cell (Tconv) immunophenotypes were selected. Each of these pairs was age- and gender-matched (Age band range: 25-45, 10 females, 10 males) and were stabilised for the majority of known T1D-associated alleles.

2.8.1.3 Monozygotic twin study

T1D-discordant monozygotic twin pairs were recruited from the British Diabetic Twin Study at Queen Mary University of London. This cohort currently consists of 546 monozygotic and dizygotic twin pairs residing

within the U.K., who were referred by their physicians (Beyan *et al.*, 2012). All twins are Caucasian and were enrolled between 1967 and 2012. The type of diabetes they exhibited was determined following the standard guidelines (Gavin *et al.*, 2003) and any cases in which this definition was unclear, were excluded. T1D was classified according to the control of diabetes with insulin from time of diagnosis and diabetic ketoacidosis or marked ketonuria at time of clinical onset. Originally, monozygosity was confirmed in each twin pair either by their self-reported status as twins or blood group typing; a method of confirming identity with no more than a 3% chance of error (Barnett *et al.*, 1981). More recently monozygosity has been proven using DNA fingerprinting (personal communication-(Hawa, 2012)).

Twins were selected according to a long period of discordance (mean number of years: 30.3 ± S.D.: 9.0) following diagnosis of the index twin (the first twin to be diagnosed). Besides insulin or insulin analogues, the only other medication used by the diabetic twins was Simvastatin (in twin pairs one and four) and antihistamines (in twin pairs one and three) (personal communication-(Hawa, 2013)). The only medication used by the non-diabetic twins, was cocodamil, for tennis elbow (in twin pair one) and ventolin and becotide for asthma (in twin pair three) (personal communication-(Hawa, 2013)). The absence of diabetes in the non-diabetic co-twins was assured previously by oral and blood glucose tolerance tests, with results being less than 7.0 mmol/l. Together with the lack of islet autoantibodies (glutamic acid decarboxylase 65 (GAD65) antibody (GADA)

and insulinoma-associated protein 2 (IA-2) antibody) the likelihood of the co-twins developing T1D at a future time point was minimal (less than 2%). Non-diabetic control individuals were recruited from the local cohort of blood donors at King's College London. This control cohort was gender-, age- and HLA-DRB1-matched to the twin cohort (Twin donors: two males, two females, mean age: 43.5 ± S.D.: 12.4. Control donors: three males, three females, mean age: 41.67 ± S.D.: 11.24.).

2.8.2 Isolation of peripheral blood mononuclear cells (PBMC) from whole blood

PBMC were isolated from whole blood by density centrifugation. For this, the blood was layered on top of at least half its volume of lymphoprep and centrifuged at 400x g for forty minutes at room temperature. The PBMC layer from each tube was carefully removed and washed with RPMI P/S/F by centrifuging at 300x g for ten minutes, at room temperature. The pellets were then combined and washed for a final time in FACS Buffer at 200x g for ten minutes after the cells were counted using a haemocytometer.

2.8.3 Measurement of the phosphorylation of STAT5a

Dr. Timothy Tree and Dr. Jennie Yang measured the phosphorylation of STAT5a (pSTAT5a) in T cells for the Protective P1 haplotype study (Chapter Five). The BD Phosflow™ kit was used for this purpose with the implementation of the manufacturer's protocol. This involved incubating

10^6 PBMC with 0.1-100 IU/ml of IL-2 for ten minutes at 37°C. They were then fixed with Phosflow Buffer 1 followed by permeabilisation with Permeabilisation Buffer III. The PBMC were stained with anti-CD4-FITC, anti-CD25-PE (M-A251), anti-CD127-PerCP-Cy5.5, anti-CD45RA-eFluor450 and anti-pSTAT5a-AF647 antibodies for thirty minutes in the dark at 4°C. The cells were washed twice by centrifugation at 400xg for 10 minutes then analysed using a FACSCanto II.

To validate earlier findings, simultaneous detection of pSTAT5a and FOXP3 was conducted in a separate cohort of samples by Dr. Jennie Yang. For this assay, 500µl of fresh blood was incubated together with 500µl X-VIVO 1% media containing 0.3, 1, 4, 10 or 100 IU/ml of IL-2 for ten minutes at 37°C. BD Lyse/Fix buffer, preheated to 37°C, was then added to fix the cells, followed by a ten minute incubation at 37°C. The cells were then washed at 600xg for eight minutes at room temperature, prior to being re-suspended and washed in Staining Buffer at 4°C for five minutes at 600xg.

The cells were permeabilised following incubation on ice with pre-cooled 100% methanol for twenty minutes. The cells were washed four times with Stain Buffer at 800xg for five minutes. The cells were re-suspended in 50µl of Stain Buffer and stained with anti–CD4-AF700, anti–CD25-APC, anti–CD45RA-PB, anti–FOXP3-PE, and anti–STAT5a-AF488 antibodies for one hour at 4°C. Following two washes in Staining Buffer as before, the cells

were analysed using a FACSCanto II.

2.8.4 Cell sorting

Tconv and Treg populations were isolated by cell sorting using a FACS Aria. Following the isolation of PBMC, the cells were re-suspended in FACS Buffer and following the addition of antibodies, were at a final concentration of 10^7/100µl. For the genotype-immunophenotype studies (Chapters Four and Five), the cells were stained with anti-CD127-PerCP-Cy5.5, anti-CD4-eFluor450, anti-CD25-PE (both the 2A3 and M-A251 clones), anti-CD14-APC-Cy7 and anti-CD45RA-AF700 antibodies. To the isotype control sample, anti-CD4 eFluor450, anti-CD14-APC-Cy7 and anti-CD45RA-AF700 antibodies were added along with mouse IgG1 PerCPCy5.5 and PE. The monozyogotic twin samples (Chapters Six and Seven) were stained with anti-CD4-QDot605, anti-CD14-AF488, anti-CD19-AF488, anti-CD25-PE (only the M-A251 clone) and anti-CD127-PerCP-Cy5.5 antibodies. To the isotype control sample, anti-CD4-QDot605, anti-CD14-AF488, anti-CD19-AF488 with mouse IgG1 PerCPCy5.5 and PE were added.

The cells were incubated with the antibodies for thirty minutes in the dark at 4°C and were then washed by centrifugation in FACS Buffer at 400 x g for ten minutes at 4°C and re-suspended in Re-suspension Buffer. The cells were passed through a FACS Aria II and were gated on the lymphocyte and monocyte populations according to their position on a FACS plot of SSC-A

against FSC-A. CD4+ Tconv were selected by gating on CD4+ CD14- cells. The Tconv population was CD127hi and CD25-/+ and the Treg population was sorted according to low expression of CD127 and high expression of CD25. Examples of the gating strategies used in the studies of this thesis are shown in the relevant results chapters.

Cells were sorted into FACS tubes filled with Collection Buffer that had been incubated at 37°C for at least ninety minutes prior to use. Running a small number of the cells back through the FACs Aria checked purity of the sorted cells. Purity was at least 99%. Following the sort, the cells were pelleted at 400x g for twenty minutes at room temperature, re-suspended in X-VIVO 5% and counted using a haemocytometer.

2.8.5 Staining of FOXP3 in Tregs

FOXP3 staining was conducted by Ms Garima Garg, as part of the genotype-immunophenotype studies (Chapters Four and Five). Tregs were fixed and permeabilised using the Biolegend FOXP3 Fix/Perm Buffer Set, according to the manufacturer's instructions. The Tregs were either taken directly from the sort or were seeded in X-VIVO 5% at 10^4 cells/well of a 96-well U-bottom plate, in a total of 0, 2 or 20 IU/ml of IL-2 at 37°C for forty-eight hours. The cells were washed with FACS Buffer at 400x g for ten minutes, re-suspended in 1x Fix/Perm Buffer and incubated in the dark at room temperature for twenty to thirty minutes.

Following washing in 1x Permeabilisation Buffer at 400x g for five to ten minutes, the cells were incubated in the dark at room temperature, for fifteen to thirty minutes in 1x Permeabilisation Buffer. Anti-FOXP3-AF647 and anti-Helios-FITC antibodies were then added and the samples were incubated in the dark for thirty minutes at room temperature, before washing with FACS Buffer. The samples were then analysed using a FACS Canto II.

2.8.6 Apoptosis assay

The level of apoptosis in sorted Tregs was assessed by Ms Garima Garg as part of the genotype-immunophenotype studies (Chapters Four and Five). The Tregs were either taken directly from the sort or were seeded in X-VIVO 5% at 10^4 cells/well of a 96-well U-bottom plate, in a total of 0, 0.2 or 2 IU/ml of IL-2 at 37°C for forty-eight hours. The cells were washed with PBS at 400x g for ten minutes, and following re-suspension in PBS, YO-PRO® -1 was added to the cells, which were then incubated in the dark for twenty minutes. 7-AAD was then added to the cells and all samples were incubated on ice for ten minutes in the dark. The samples were then analysed using a FACS Canto II.

2.8.7 Expansion of Treg cell lines (Standard Tregs)

The expanded Treg cell line; Standard Tregs, used as part of the genotype-immunophenotype studies (Chapters Four and Five) had been generated

previously by Dr. Jennifer Lawson. PBMC were isolated from a healthy individual recruited from the local cohort of donors, by density centrifugation, as described in Section 2.8.2. The cells were stained with anti-CD3-FITC, anti-CD4-APC, anti-CD8-PE-Cy7 and anti-CD25-PE antibodies, following which the Tregs were isolated by cell sorting, in the same manner described in Section 2.8.4. The Tregs isolated were CD3+ CD4+ CD8- CD25hi. These cells were then seeded at 10^5/well in a 48 well plate with irradiated mixed donor (allogeneic) PBMC (feeder cells) at 10^6/well and expanded with 4µg/ml Phytohaemagglutinin (PHA) and 600 IU IL2/ml. Media containing IL-2 was replaced every two days and the cells passaged as required. Following ten days of culture, the cells were re-stimulated with irradiated feeder cells and PHA as described above.

After a further ten days in culture, cells were frozen in aliquots of three million cells. For this, cells were pelleted by centrifugation at 400xg for 10 minutes at 4°C, before being re-suspended in the appropriate volume of FCS. An equal volume of FCS containing 20% DMSO was then added dropwise to the cells. The cells in suspension were added to pre-chilled cryovials and were placed inside a CoolCell®, which was immediately cooled in the -80°C freezer. The cryovials were transferred to a liquid nitrogen storage tank twenty-four hours later.

2.8.8 *In vitro* co-culture suppression assay

Total or subpopulations of Tconv were co-cultured in X-VIVO 5% in 96-well U bottom plates at $2.5x10^3$/well with 0-$2.5x10^3$/well of autologous Tregs or Standard Tregs. For the monozygotic twin study, crossover co-cultures whereby the Tregs from one twin were co-cultured with Tconv from the other twin and *vice versa*, were also set up. The cells were polyclonally stimulated with either Dynabeads® (magnetic beads coated with anti-CD3 and anti-CD28 antibodies) at bead:cell ratios of 1:1 or 1:2.5 (two-cell Treg suppression assay) or plate-bound anti-CD3 antibody at 0.25μg/ml or 0.5μg/ml together with soluble 5μg/ml anti-CD28 antibody and $2.5x10^4$/well of irradiated accessory cells (ACs) (three-cell Treg suppression assay). For the three-cell suppression assay, plates were pre-coated with the anti-CD3 antibody by adding 50μl/well of this antibody and incubating the plate overnight at 4°C, prior to washing three times with 1xdPBS. Also, following addition of the cells, plates were centrifuged at 400x g for ten minutes at room temperature, to ensure the monocytes did not adhere to the sides of the wells. The plates were then cultured at 37°C, 5% CO_2. All conditions were set up in triplicate.

After five days, 100μl of supernatant was removed from each of the wells and stored at -80°C, for later cytokine analysis. 100μl of fresh X-VIVO 5% containing 0.5μCi of 3[H]-thymidine was added to each well for the remaining eighteen hours of culture. The cultures were then harvested onto

fibreglass filter mats using a Filtermate Harvester. Once dry, the filter mats were placed inside sample bags and saturated with Betaplate scintillation fluid and the bags sealed. The incorporation of 3[H]-thymidine was measured using a Microbeta Trilux machine. The level of suppression was then calculated using the formula: % suppression= 100-(counts per minute (cpm) in the presence of Tregs ÷ cpm in the absence of Tregs) x 100).

2.8.9 Tr1-generation assay

96-well flat-bottom plates were coated with 50µl of 1xdPBS containing anti-CD3 antibody with either anti-CD28 antibody or anti-CD46 antibody (all at 2.5µg/ml) overnight at 4°C. The plates were washed three times with 1xdPBS, before Tconv from the sort were seeded at $1.5x10^5$/well in RPMI 10%. IL-2 was added to the respective wells to produce final concentrations of 2, 10, 20 or 50 IU/ml in RPMI 10%. The plates were spun at 200xg for two minutes before they were incubated at 37°C, 5% CO_2. Supernatants were taken three days later and were stored at -80°C for later cytokine analysis.

2.8.10 ELISAs

Sandwich enzyme-linked immunosorbent assay (ELISA) kits supplied by Biolegend were used to detect and quantify IFNγ and IL-10 in cell supernatants. 96-well MaxiSorp Immuno plates were coated with 50µl/well

of anti-IFNγ or anti-IL-10 capture antibody in Coating Buffer then incubated overnight at 4°C. The following day, the plates were washed four times with ELISA Wash Buffer, before non-specific binding was blocked by addition of Assay Diluent to each well. The plates were incubated at room temperature on a plate shaker for one hour, prior to being washed as before, after which, standards and samples diluted in Assay Diluent were added to the wells. The plates were incubated for a further two hours at room temperature on the plate shaker. After washing, anti-IFNγ or anti-IL-10 detection antibodies diluted in Assay Diluent were added, followed by another one-hour incubation on the plate shaker. Once the plates had been washed as before, Avidin-HRP, diluted in Assay Diluent was added to each well. The plates were incubated for thirty minutes at room temperature with shaking, before being washed five times, and the substrate was added. The substrate was made up using a Substrate Reagent Pack. Equal volumes of stabilised hydrogen peroxide and stabilised TMB were mixed, prior to addition to the plate. After developing, a 2M solution of sulphuric acid was added to each well to stop the reaction and the absorbance measured at 450nm-570nm by a plate reader. The unknown concentrations of the samples were calculated from standard curves drawn using GraphPad Prism software.

2.8.11 Statistics

Statistical analyses were conducted using GraphPad Prism. Before statistical tests were applied, normal (Gaussian) distribution was assessed

by the use of three normality tests; the Kolmogorov-Smirnov test, the D'Agostino and Pearson omnibus normality test and the Shapiro-Wilk normality test. Data was deemed parametric if all three tests were passed. The pairs selected for the genotype-immunophenotype studies (Chapters Four and Five) and the monozygotic twin pairs (Chapters Six and Seven) were treated as paired samples. One-way ANOVAs were conducted when more than two sets of data were compared and correlations were assessed by linear regression. Data was considered to be significant if p values were less than 0.05.

Chapter Three: Development of experimental procedures to examine regulatory T cell function and the IL-2 signalling cascade

3.1 Introduction

Before commencing any of the subsequent studies it was imperative that the experimental procedures were fully optimised before utilisation; thus ensuring the assays finally implemented were robust, reliable and gave reproducible results. Another essential quality of the assays was the ability to discriminate between the individuals tested. It has been noted by others that measurement of the suppressive function of Tregs can show considerable variation, which is dependent upon the strength of the stimulation conditions used (Baecher-Allan *et al.*, 2001, Viglietta *et al.*, 2004, Lindley *et al.*, 2005). Therefore the optimal conditions for the assays in this thesis were sought, which could repeatedly show differences between healthy control donors, with the reasoning that these would also highlight variations between the subjects tested in this thesis.

3.1.1 Optimisation of the Treg suppression assay

By implementing the classical 'Shevach'-style *in vitro* co-culture suppression assay (Thornton and Shevach, 1998) (which involves co-culturing Tconv and Tregs and using the resulting suppression when compared to

stimulated cultures of Tconv alone, as a measure of Treg function) several studies (including previous work conducted in this laboratory) have identified a defect in Treg suppressive function in T1D compared to non-diabetics (Lindley *et al.*, 2005, Lawson *et al.*, 2008, Brusko *et al.*, 2005, Jana *et al.*, 2010, Glisic-Milosavljevic *et al.*, 2007b) (see Chapter One, Section 1.14). The aim of this thesis was to determine whether this defect is genetically determined or could be a consequence of the disease itself. (Although it should be noted other factors may be implicated e.g. epigenetics.).

The 'Shevach' suppression assay was also adopted for this thesis but first underwent optimisation ensuring it was fully suited to its purpose for each of the following studies. The main reason being that in comparison to earlier studies conducted in this laboratory, this thesis utilised fluorescence activated cell sorting (FACS) instead of magnetic activated cell sorting (MACS) (Lindley *et al.*, 2005, Lawson *et al.*, 2008). Isolation of cells by FACS has been clearly demonstrated to result in purer populations of cells than MACS (Tree *et al.*, 2006).

To assess the aim of this thesis, two genotype-immunophenotype studies were first conducted (Chapters Four and Five). Each of these examined the implications of two T1D-associated *IL-2RA* SNPs (discussed in detail in Chapter One, Section 1.13) on Treg function. The first SNP assessed was SNP.286 and the presence of a protective allele at this locus is associated

with lower percentages of CD25+ CD4+ naïve Tconv compared to the susceptible allele (Dendrou *et al.*, 2009) (see Chapter Four). The second SNP examined was SNP.495 a protective allele at which is associated with the presence of higher levels of CD25 on CD4+ memory Tconv and higher production of IL-2 by these cells, when activated, compared to the susceptible allele (Dendrou *et al.*, 2009) (see Chapter Five).

To examine each of these SNPs, a pair-wise analysis was conducted by comparing non-diabetic donors with the protective allele at the SNP of interest, with those with the susceptible allele (see Chapter Four, Section 4.1 and Chapter Five, Section 5.1.3 for a more detailed description). As neither of these SNPs influences the phenotype of APC (Dendrou *et al.*, 2009), a two-cell suppression assay was used, to examine the direct action of Tregs on Tconv, in the absence of APC. These assays were stimulated with Dynabeads® (magnetic beads coated with anti-CD3 and anti-CD28 antibodies), which allowed cells to be stimulated without APC co-stimulation (Oberg *et al.*, 2006). In the latter half of this thesis, Treg function and Tr1 cell generation was compared in pairs of T1D-discordant monozygotic twins and non-diabetic non-twin control donors (see Chapters Six and Seven). To examine Treg function in these studies, both a two-cell and a three-cell suppression assay (with accessory cells (AC)) was used. Table 3.1 details how the two-cell and three-cell suppression assays compare. Table 3.2 summarises the relevant details of the subsequent studies comprising this thesis.

		2-cell suppression assay	3-cell suppression assay
Cells used	Tregs	√	√
	Tconv	√	√
	ACs	x	√
Stimuli	Dynabeads®	√	x
	Anti-CD3/CD28 antibodies	x	√

Table 3.1 Comparison of the two-cell and three-cell in vitro cell-culture suppression assays

Chapter	Research question	Subjects examined	Cell populations analysed	Assays conducted
Four	How does SNP.286 influence naïve Tconv resistance to Treg suppression?	Local control donors	-CD4+ Tconv (memory, naïve, CD25-/CD25+ naïve) -Autologous Treg -Standard Treg	Two-cell suppression assay
Five	How does SNP.495 influence memory Tconv resistance to Treg suppression?	Non-diabetic donors from the Cambridge BioResource cohort	-CD4+ Tconv (memory, naïve, CD25-/CD25+ naïve) -Autologous Treg -Standard Treg	Two-cell suppression assay
Six	Is defective Treg function a cause or a consequence of T1D?	T1D-discordant monozygotic twins Local control donors	-CD4+ Total Tconv -Autologous Treg -B cells & monocytes	Two-cell and three-cell suppression assay
Seven	Is defective IL-2 signalling a cause or a consequence of T1D?	T1D-discordant monozygotic twins Local control donors	-CD4+ Total Tconv	Tr1 generation assay

Table 3.2 Details of the studies comprising this thesis

As explained in Chapter One, Section 1.9, defective Treg function could either result from an intrinsic defect in the Tregs themselves or be due to increased Tconv resistance to Treg suppression. In T1D, it has been shown the CD4+ CD25- Tconv are less sensitive to Treg suppression (although in some patients a decrease in the function of Tregs was also seen) (Lawson *et al.*, 2008, Schneider *et al.*, 2008). Therefore, in addition to measuring the suppressive capacity of autologous Tregs in this thesis, Standard Tregs (an expanded Treg cell line from a third party donor) (generated by J. Lawson) were also co-cultured with donor Tconv populations as part of the genotype-immunophenotype studies (see Chapters Four and Five) to assess their sensitivity to Treg suppression. By using the same batch of Standard Tregs at the same passage for each of these donors, these cells served as a constant factor in each pair tested. It should be noted that although the cells in Standard Treg co-cultures were subject to allogeneic activation, the polyclonal stimulation added to these cultures would presumably have acted as a far stronger stimulus. As part of the study involving monozygotic twins, crossover co-cultures were used to assess Tconv sensitivity to Treg function (see Chapter Six, Section 6.2.8).

Finally, the stimuli strengths administered to the two-cell and three-cell suppression assays required optimisation. It was essential that a good dynamic range of stimuli be used to be able to identify differences between donors in terms of Treg function. This is explained more thoroughly in Section 3.3.2. Also, the Treg suppression assays had to show reproducibility

to ensure the results were reliable.

3.1.2 Optimisation of the Tr1-generation assay

In addition to nTregs, several subsets of adaptive Tregs exist, which are induced in the periphery (see Chapter one, Section 1.2). One such population are Tr1 cells (Groux *et al.*, 1997, Kemper *et al.*, 2003) characterised by production of high levels of IL-10 with equal or higher levels of IFNγ (Cardone *et al.*, 2010) (see Chapter One, Section 1.5). Kemper and colleagues demonstrated that Tr1 cells could be generated from CD4+ Tconv cells by stimulation with anti-CD3 and anti-CD46 antibodies in an IL-2 dose-dependent manner (Cardone *et al.*, 2010). This method of generating these cells was of interest in this thesis, as the IL-2 signalling cascade has been reported to be deficient in T1D (Kaye *et al.*, 1986, Zier *et al.*, 1984, Roncarolo *et al.*, 1988, Long *et al.*, 2010, Long *et al.*, 2011) (see Chapter One, Section 1.14.5). However, it is not clear whether this defect is a cause or consequence of the disease.

To investigate the response to IL-2, a Tr1 generation assay was optimised to examine not just the production of these cells from CD4+ Tconv from diabetics and controls, but also to compare Tr1 generation in diabetics with their non-diabetic twins. Whilst based upon the assay described by Kemper and colleagues, it required optimisation for use in this thesis as the authors isolated cells by MACS instead of FACS and also used different clones of

antibodies to stimulate the Tconv (Cardone *et al.*, 2010). In this chapter statistical analyses were performed as described in Chapter Two, Section 2.8.11 and are given in each figure.

3.2 Results

3.2.1 Isolation of Tregs and CD4+ Tconv from PBMC

FACS was implemented in this thesis to isolate the cells of interest and was ideal for isolating the different Tconv populations needed for the genotype-immunophenotype studies conducted in Chapters Four and Five. Also by using FACS, the Treg population could be isolated using the markers CD25 and CD127 in combination. Tregs express high levels of CD25 with low expression of CD127 (see Chapter One, Section 1.2) and higher and purer percentages of Tregs can be obtained when the CD127 marker is used in unison with CD25, compared to CD25 alone (Liu *et al.*, 2006b). For the genotype-immunophenotype studies (Chapters Four and Five) Tregs, total CD4+ memory Tconv, total CD4+ naïve Tconv and the CD25- and CD25+ naïve Tconv subpopulations were isolated for use in the Treg suppression assay (Figure 3.1).

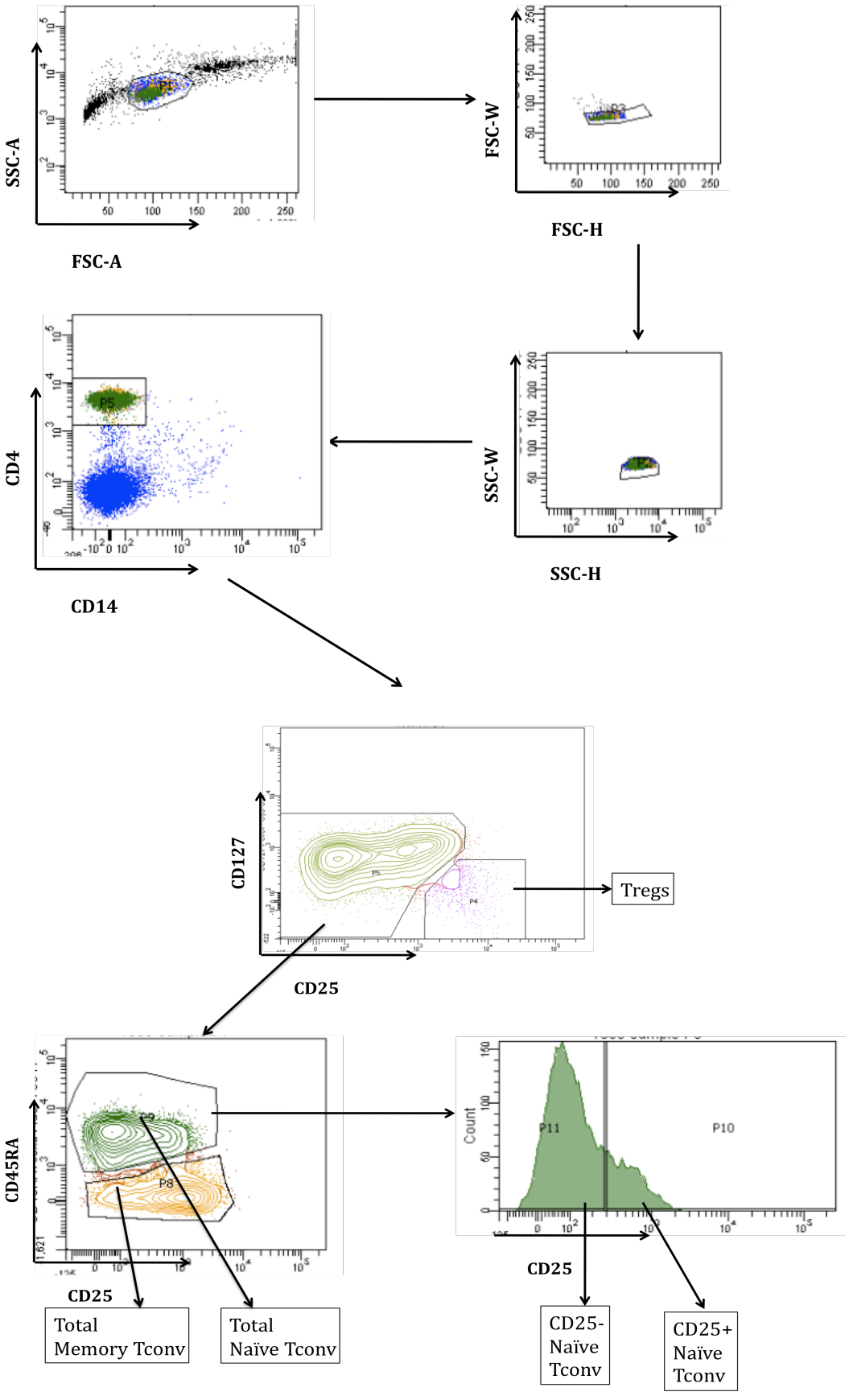
SSC-A
FSC-A
FSC-W
FSC-H
CD4
CD14
SSC-W
SSC-H
CD127
CD25
Tregs
CD45RA
CD25
Count
CD25
Total Memory Tconv
Total Naïve Tconv
CD25- Naïve Tconv
CD25+ Naïve Tconv

Figure 3.1 Gating strategy for the isolation of Tregs, total memory, total naïve and naïve CD25-/+ Tconv for the genotype-immunophenotype studies

The gating used to isolate cells for the genotype-immunophenotype studies is shown. Firstly, lymphocytes were gated and doublets removed. After gating on CD4+ T cells, the Tconv were selected by high expression of CD127 and negative to intermediate expression of CD25. Tregs were isolated according to high expression of CD25 correlating with low levels of CD127. Memory Tconv were isolated according the absence of CD45RA, whilst naïve Tconv were isolated according to its presence. After the required number of naïve Tconv was collected, the remaining naïve Tconv population was divided according to the absence or presence of CD25.

3.2.2 Optimisation of the stimuli strengths used in the two-cell suppression assay to study the effects of two T1D-associated *IL-2RA* SNPs on Treg function

To study the two *IL-2RA* SNPs, the function of Tregs *in vitro* was assessed by a two-cell suppression assay optimised for this purpose (see Chapter Two, Section 2.8.8 and Chapters Four and Five). A number of stimuli strengths were tested so that those resulting in acceptable levels of proliferation with measurable levels of suppression (in all subjects tested) could be selected. The signal strength administered by Dynabeads® was first optimised in memory Tconv, by examining a range of Dynabeads®:cell (bead:cell) ratios; 1:1, 1:2.5 and 1:5. Memory Tconv were stimulated either alone or in co-cultures with Tregs, at Tconv:Treg ratios of 1:1, 2:1, 4:1 and 8:1. To ensure the Treg population was anergic, this cell population was also cultured alone and subjected to the same stimuli. As shown in Figure 3.2, the stronger the stimulus, the higher the level of proliferation. Higher levels of suppression were seen with a bead:cell ratio of 1:2.5 than 1:1.

Using Dynabeads® at 1:5 provided too weak a stimulus to produce satisfactory levels of Tconv proliferation when these cells were cultured alone, with levels barely above background. Although suppression for this stimulus is shown in Figure 3.2B, this stimuli strength is clearly not suitable to produce the robust levels of proliferation needed to quantify suppression accurately in all subjects. For this reason, the bead:cell ratio of 1:5 was omitted from further studies. Dose-dependency of Treg-mediated

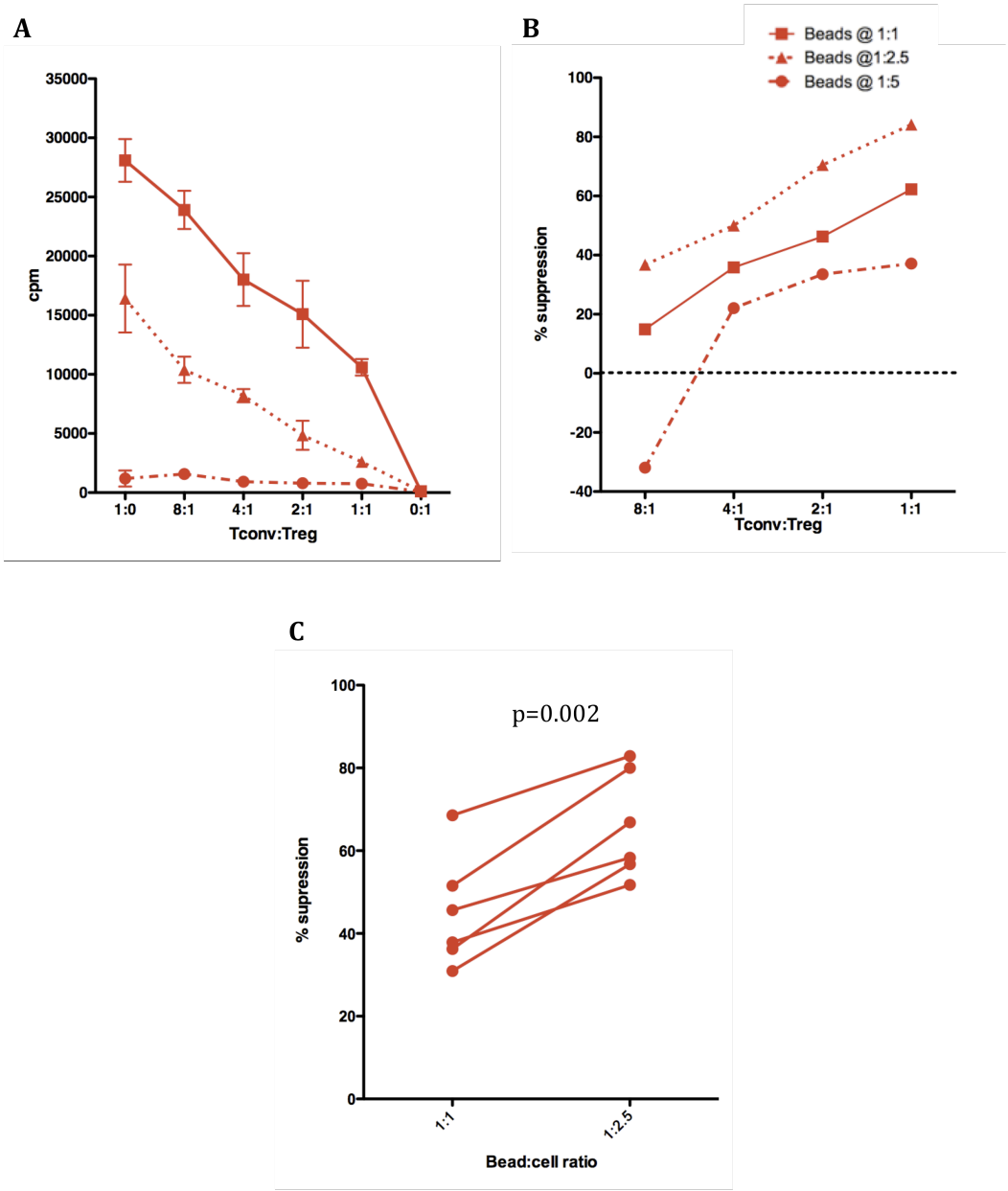

Figure 3.2 Proliferation and suppression is linked to signal strength and Tconv:Treg ratio

A range of Dynabeads®:cell ratios were tested to optimise the level of proliferation and suppression in the majority of donors tested. The bead:cell ratios examined were 1:1 ( ), 1:2.5 ( ) and 1:5 ( ). Figure A shows the level of proliferation of memory Tconv from donor T011, either alone or in co-

culture with autologous Tregs at the different Tconv:Treg ratios shown. Proliferation is shown as mean counts per minute (cpm) of 3[H]-thymidine in triplicate wells and error bars show standard deviation. Figure B shows the mean level of suppression of memory Tconv proliferation for T011, across the triplicate wells at the different stimuli strengths and at different Tconv:Treg ratios. Suppression was calculated using the formula: % suppression= 100-(counts per minute (cpm) in the presence of Tregs ÷ cpm in the absence of Tregs) x 100). The data shown here are representative of all ten donors tested. Figure C shows the levels of suppression of memory Tconv at a ratio of Tconv:Treg of 1:1 when stimulated with beads at 1:1 and 1:2.5 for six out of the nine other donors tested. An interconnecting line shows data from each donor. Data is analysed by a two-tailed paired t test.

suppression was seen in most individuals, with an increase in suppression correlating with the presence of more Tregs.

As mentioned in Section 3.1.1 the Treg suppression assay needed to demonstrate reproducibility. Figure 3.3 shows the level of suppression measured in Treg suppression assays set-up on two separate occasions, following two independent blood draws, with cells from a pair of healthy control donors (denoted T012 and T016). The cells were stimulated with bead:cell ratios of 1:1 and 1:2.5. Again, higher levels of suppression are seen when the cells are stimulated with a bead:cell ratio of 1:2.5, than 1:1, however the decrease in suppression at this higher stimulus is more striking in co-cultures from T016, than T012. The effect is that there is little contrast in suppression between the two donors at 1:2.5, but there is a discernable difference at 1:1. This highlights the need for a dynamic range of stimuli allowing differences between individuals to be identified. The differences in suppression between these two donors at these signal strengths are also shown to be reproducible on two separate occasions, thus demonstrating the utility of the assay.

Suppression assays examining inhibition of naïve Tconv proliferation were also tested at 1:1 and 1:2.5 (Figure 3.4). These assays also showed acceptable levels of proliferation and measurable levels of suppression for all donors tested, with higher levels of suppression again being seen at 1:2.5 compared to 1:1. Co-cultures at Tconv:Treg ratios of 1:1 and 2:1 showed the

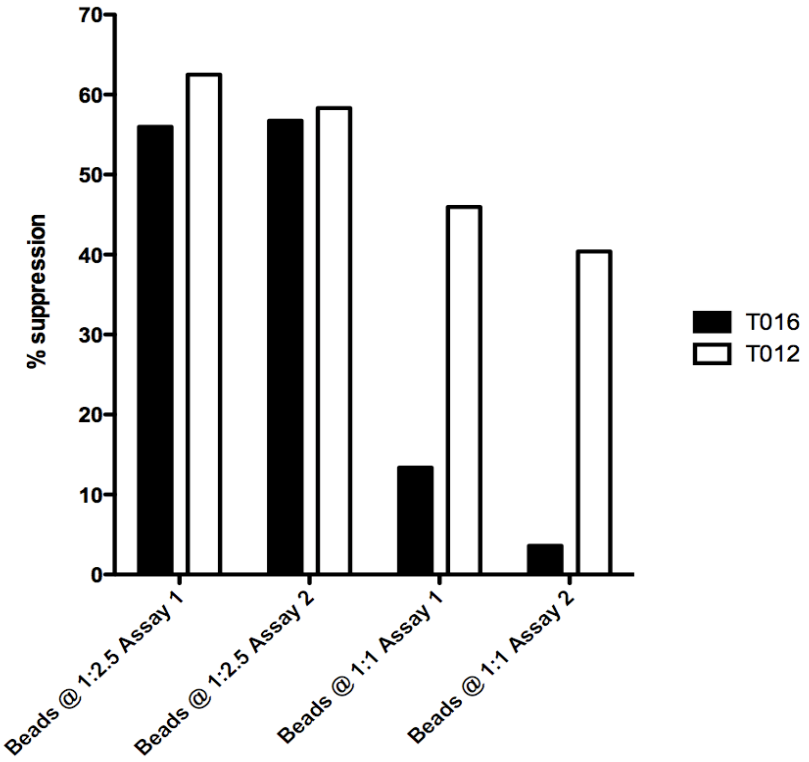

Figure 3.3 Importance of the signal strength administered to the Treg suppression assay in generating reproducibility and identifying differences between individuals

The mean level of suppression of memory Tconv proliferation when in co-culture with equal numbers of autologous Tregs is shown for two healthy control donors, T012 (▭) and T016 (■). These suppression assays were conducted on two separate occassions. The percentage suppression is calculated from the mean level of suppression of Tconv cells in triplicate wells, using the formula: % suppression= 100-(counts per minute (cpm) in the presence of Tregs ÷ cpm in the absence of Tregs) x 100). Co-cultures were stimulated with either a bead:cell ratio of 1:1 or 1:2.5

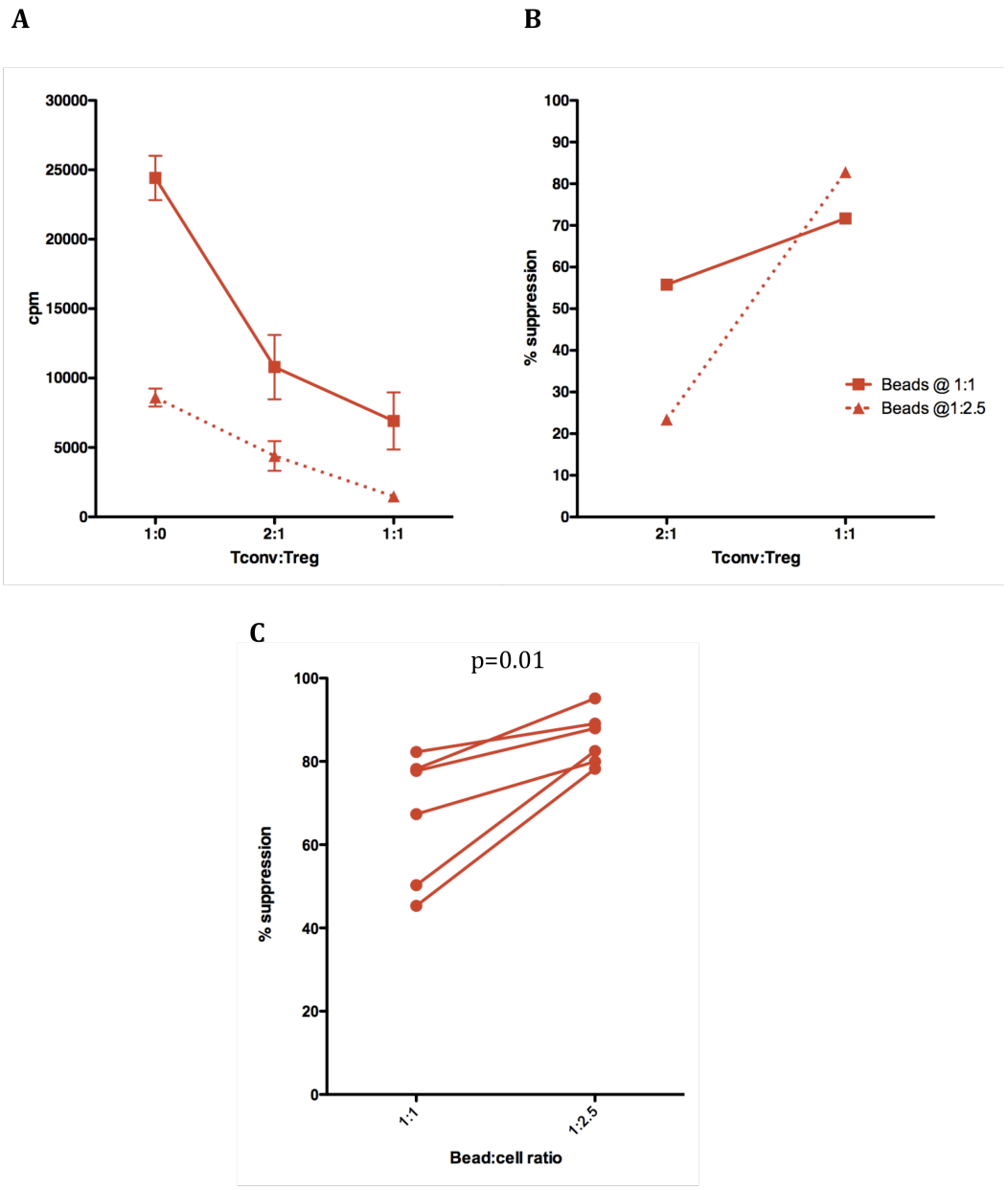

Figure 3.4 Proliferation and suppression is linked to signal strength and Tconv:Treg ratio in naïve Tconv

Proliferation of naïve Tconv (Figure A) from healthy control donor G048 when cultured alone or in co-culture with Tregs. Cultures were stimulated with bead:cell ratios of 1:1 ( )and 1:2.5 ( ). Proliferation measured as mean cpm of 3[H]-thymidine across triplicate wells. Error bars show standard

deviation. Figure B shows the mean level of suppression of naïve Tconv proliferation for G048, across the triplicate wells at the different stimuli strengths and at different Tconv:Treg ratios. Suppression was calculated using the formula: % suppression= 100-(counts per minute (cpm) in the presence of Tregs ÷ cpm in the absence of Tregs) x 100). The data shown here are representative of all ten donors tested. Figure C shows the levels of suppression of naïve Tconv at a ratio of Tconv:Treg of 1:1 when stimulated with beads at 1:1 and 1:2.5 for six out of the nine other donors tested. An interconnecting line shows data from each donor. Data is analysed by a two-tailed paired t test.

optimal and most robust levels of suppression (Figure 3.2) therefore these Tconv:Treg ratios were selected for the subsequent studies.

3.2.3 Optimisation of the Treg suppression assay utilising Standard Tregs in place of autologous Tregs

As discussed in Chapter One, Section 1.8.4 and Section 3.1.1, CD4+ CD25- Tconv from diabetics show an increased resistance to Treg suppression (Lawson *et al.*, 2008, Schneider *et al.*, 2008). Therefore, as part of the genotype-immunophenotype studies, Standard Treg co-cultures were set-up in parallel to autologous co-cultures from each donor, to compare the ability of the Tconv population to be suppressed. The Standard Tregs were generated from a healthy donor (Table 3.3) (as described in Chapter Two, Section 2.8.7) and were all CD3+ CD4+ CD8- CD25hi (Figure 3.5). An example of the proliferation of memory Tconv, naïve Tconv and CD25- and CD25+ naïve Tconv in co-culture with Standard Tregs is shown in Figure 3.6. Dose-dependency of Treg-mediated suppression of Tconv proliferation is also seen in these co-cultures as with autologous co-cultures. Very high levels of suppression can be seen with these cells. As shown in Figure 3.7, donor T036 demonstrated more than 90% suppression of Tconv proliferation at all Tconv:Treg ratios tested.

3.2.4 Comparison of autologous and Standard Treg co-cultures

To assess the suppressive capacity of Standard Tregs, proliferation in

Gender	Male
Age	44
HLA-DRB1 genotype	0301, 0306

Table 3.3 Donor specifics for the individual from which standard Tregs were generated

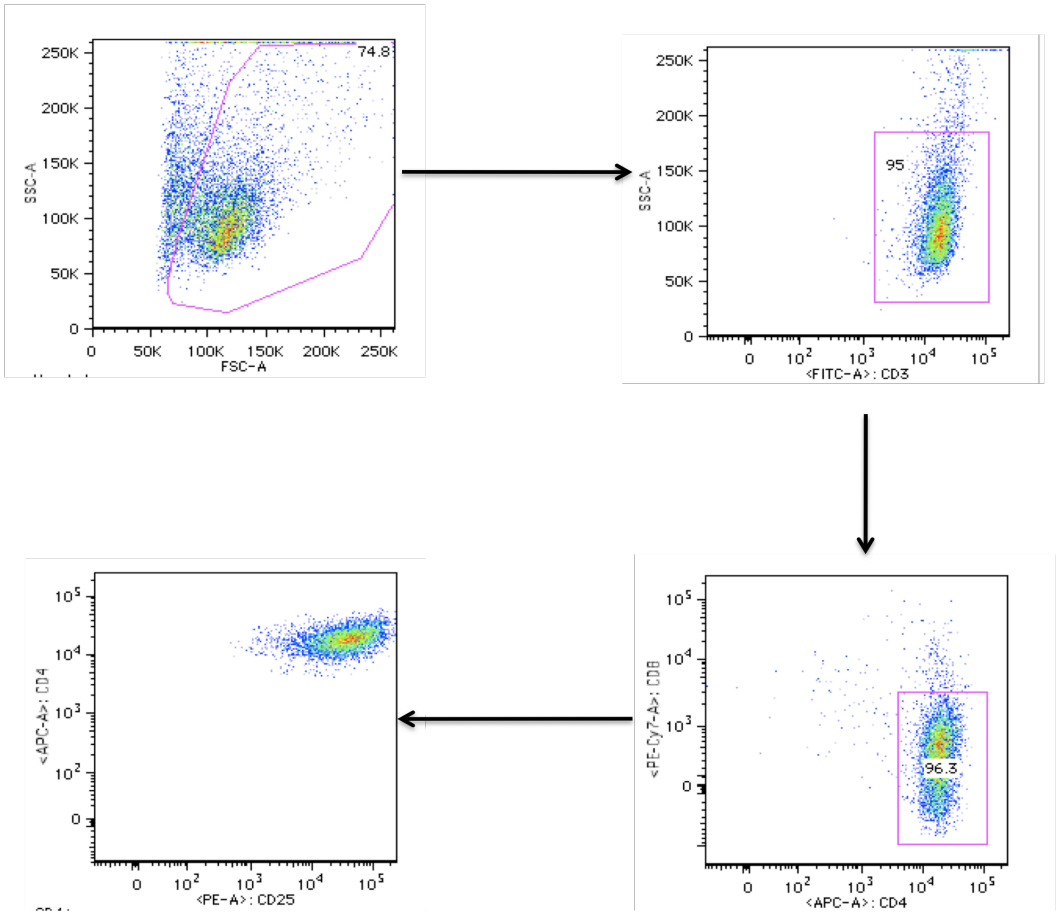

Figure 3.5 Phenotype of the Standard Tregs

Data show the expression of CD3, CD4, CD8 and CD25 on the Standard Tregs.

Data courtesy of Dr. Jennifer Lawson.

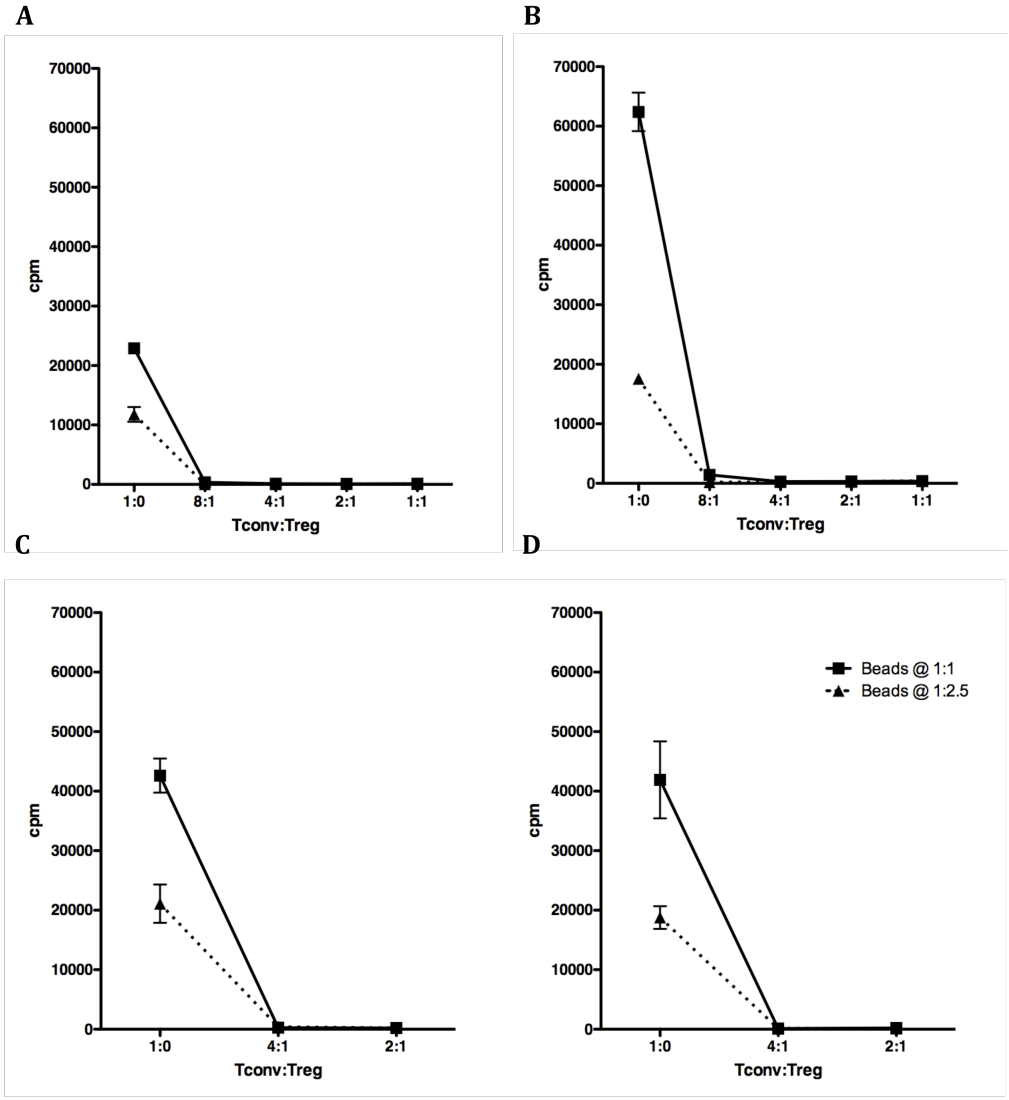

Figure 3.6 Proliferation of each of the Tconv populations in the presence or absence of Standard Tregs.

Proliferation of memory Tconv (Figure A), naïve Tconv (Figure B), CD25 negative naïve Tconv (Figure C) and CD25 positive naïve Tconv (Figure D) from donor T036, alone or in co-culture with Standard Tregs. Cultures were stimulated with bead:cell ratios of 1:1 ( )and 1:2.5 ( ). Proliferation measured as mean cpm of 3[H]-thymidine across triplicate wells. Error bars show standard deviation.

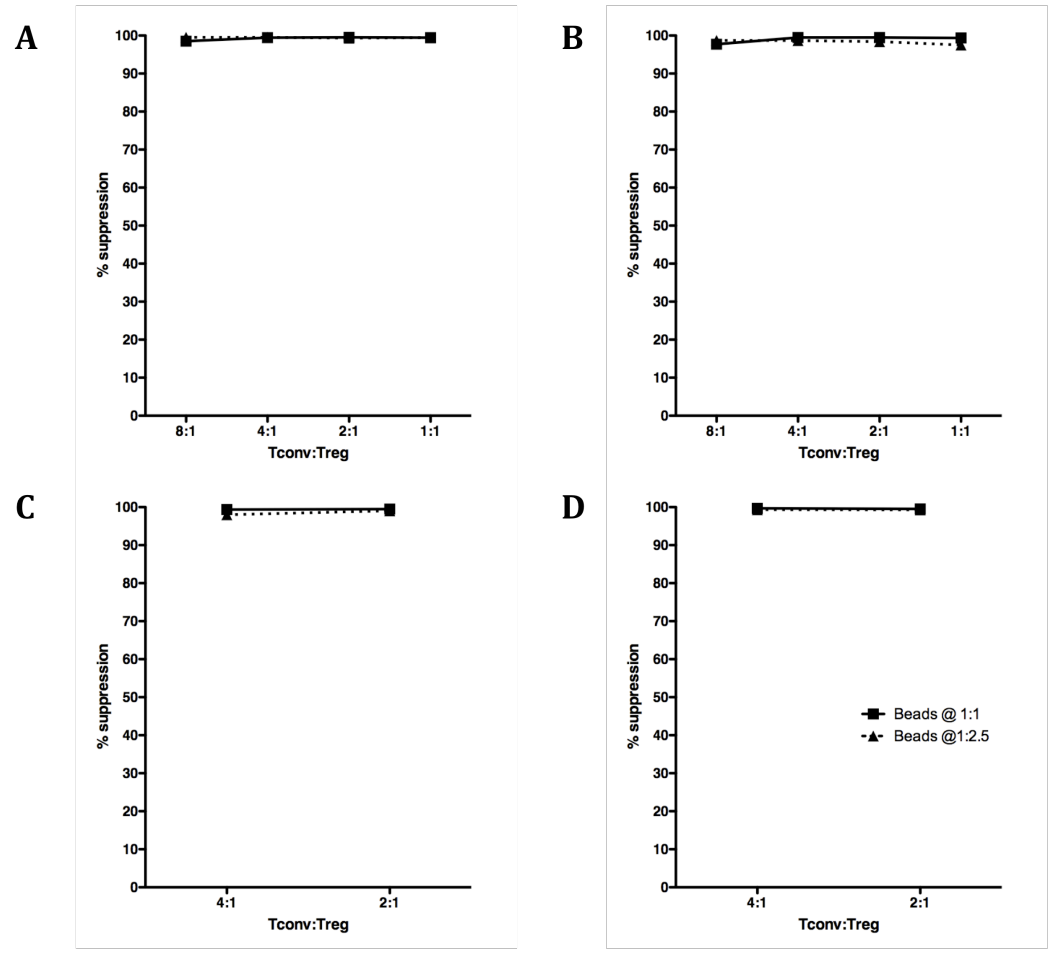

Figure 3.7 Suppression of each of the Tconv populations in the presence of Standard Tregs.

The mean level of suppression across the triplicate wells is shown for memory Tconv (Figure A), naïve Tconv (Figure B), CD25 negative naïve Tconv (Figure C) and CD25 positive naïve Tconv (Figure D) from donor T036 (figure 3.6) in co-culture with Standard Tregs. Suppression was calculated using the formula: % suppression= 100-(counts per minute (cpm) in the presence of Tregs ÷ cpm in the absence of Tregs) x 100). Cultures were stimulated with bead:cell ratios of 1:1 (■)and 1:2.5 (-▲-).

autologous and Standard Treg co-cultures from several donors was compared. Figure 3.8 shows proliferation of memory Tconv in autologous Treg and Standard Treg co-cultures from healthy control donor T002. Proliferation of stimulated Tconv alone is similar in both assays at both stimuli strengths, as expected. However, the suppressive function of Standard Tregs was so high, that no differences in Tconv resistance were discernable between subjects at a Tconv:Treg ratio of 1:1 (Figure 3.9 A-D). However, this factor could be assessed at lower ratios (Figure 3.9 E and F). Therefore, the Standard Tregs were titrated further and Tconv:Treg ratios of 1:1, 2:1, 4:1, 8:1 and 16:1 were used for Standard Treg co-cultures with memory and naïve Tconv cells when studying the *IL-2RA* SNPs. Indeed. As shown in Figure 3.7, very high levels of suppression are seen of CD25- and CD25+ naïve Tconv proliferation when Tconv: Treg ratios of 2:1 and 4:1 were observed therefore these were also titrated further to 4:1 and 16:1.

3.2.5 Optimisation of the stimuli strengths used in the two- and three-cell suppression assays to study Treg function between T1D-discordant monozygotic twins

One of the latter studies in this thesis concerns the comparison of Treg function in monozygotic twins discordant for T1D (see Chapter Six). Unlike the genotype-immunophenotype studies conducted in Chapters Four and Five, this study used the total CD4+ Tconv population as responder cells, as opposed to Tconv subpopulations. Previous studies of Treg function in T1D,

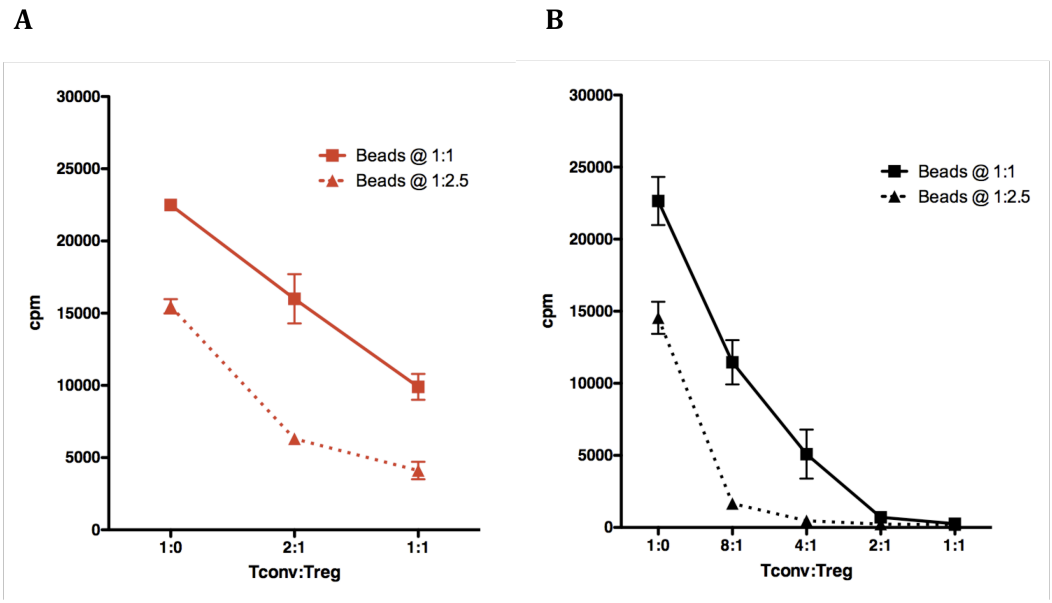

Figure 3.8 Comparison of the proliferation of memory Tconv in the presence or absence of Tregs in autologous or Standard Treg co-cultures

Proliferation of memory Tconv from donor T002 in the absence or presence of autologous Tregs (Figure A) or Standard Tregs (Figure B). Cultures were stimulated with bead:cell ratios of 1:1 (■) and 1:2.5 (▲). Proliferation measured as mean cpm of 3[H]-thymidine across triplicate wells. Error bars show standard deviation.

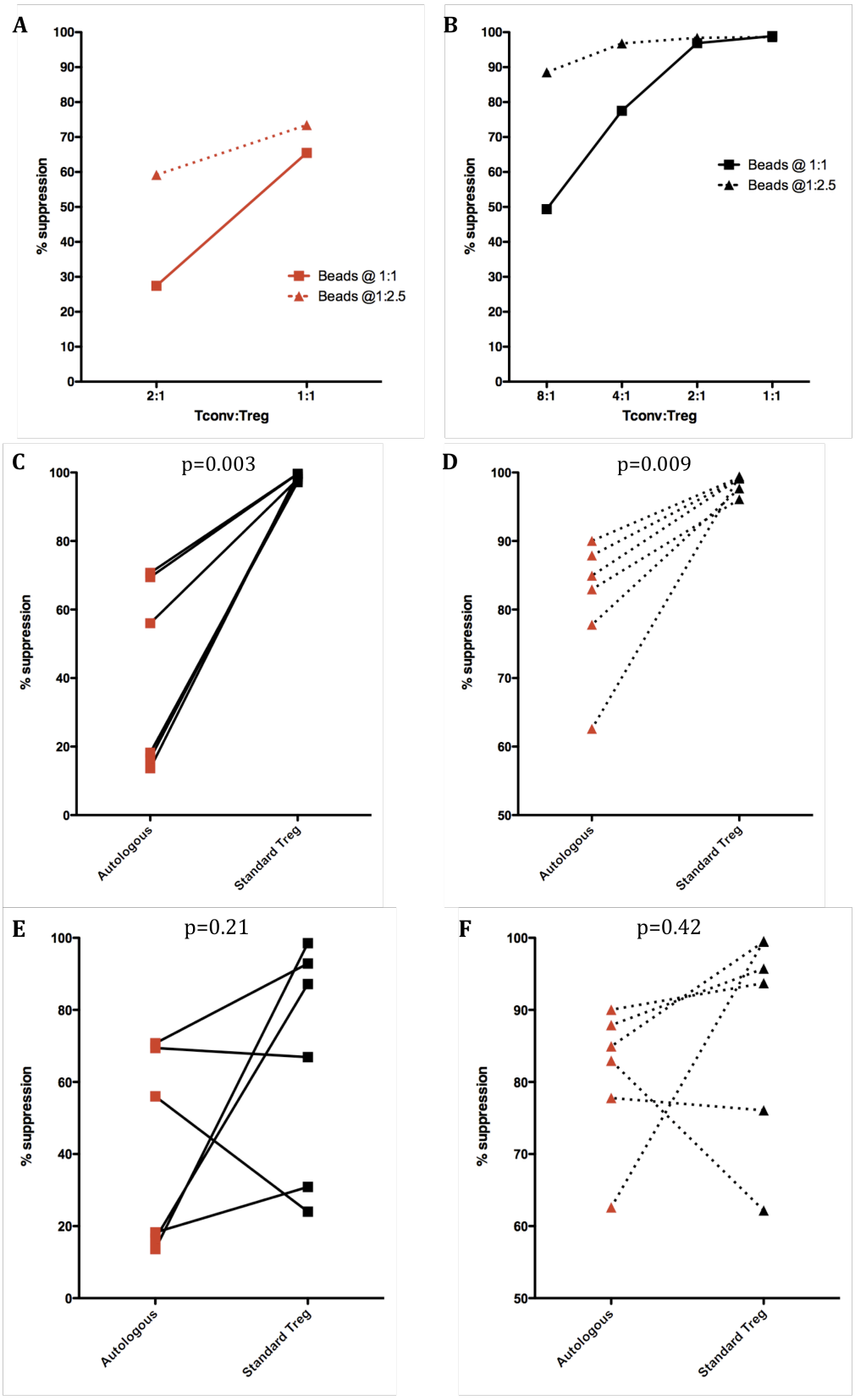

Figure 3.9 Comparison of suppression of memory Tconv in the presence of Tregs in autologous or Standard Treg co-cultures

The mean level of suppression across triplicate wells of memory Tconv from donor T002 in the presence of autologous Tregs (Figure A) or Standard Tregs (Figure B) is shown. Suppression was calculated from the proliferation measured in Figure 3.8 using the formula: % suppression= 100-(counts per minute (cpm) in the presence of Tregs ÷ cpm in the absence of Tregs) x 100). Cultures were stimulated with bead:cell ratios of 1:1 (■) and 1:2.5 (▲). The mean level of suppression of memory Tconv proliferation when in co-culture with autologous Tregs or Standard Tregs at a Tconv:Treg ratio of 1:1 (Figures C and D) or 8:1 (figure E and F) is shown for six out of nine other control donors tested. Cultures were stimulated with beads at 1:1 (Figures C and E) or beads at 1:2.5 (Figures D and F). An interconnecting line shows data from each donor. Data was analysed by a two-tailed paired t test. The data shown here are representative of all ten donors tested.

which have identified significantly lower levels of Treg suppression in diabetics, also studied the total CD4+ (CD25-) Tconv as responder cells (Lindley *et al.*, 2005, Lawson *et al.*, 2008, Brusko *et al.*, 2005, Glisic-Milosavljevic *et al.*, 2007b) and also utilised a three-cell suppression assay comprised of Tregs, Tconv and ACs. Therefore, a three-cell suppression assay was also optimised in addition to a two-cell suppression assay for use in the monozygotic twin study in this thesis. The three-cell suppression assay comprised of Tregs, total Tconv and irradiated CD14+ monocytes and CD19+ B cells as ACs (Table 3.1 illustrates how the two-cell and three-cell suppression assays compare). Figure 3.10 shows the gating strategy used to isolate the cells of interest for the monozygotic twin study.

Three different concentrations of plate-bound anti-CD3 antibody used to stimulate the cells in the three-cell suppression assay were tested: 0.25, 0.5 and 1μg/ml. As earlier studies using a three-cell suppression assay added soluble anti-CD28 antibody to their co-cultures (Brusko *et al.*, 2005, Lindley *et al.*, 2005, Lawson *et al.*, 2008) anti-CD3 antibody in the absence or presence of 5μg/ml of anti-CD28 antibody was also assessed. Previous studies in this laboratory used anti-CD28 antibody at 5μg/ml, hence why this concentration was selected for these trials (Lindley *et al.* (2005), Lawson *et al.* (2008)).

Once again, Dynabeads® generated higher levels of proliferation at a ratio of

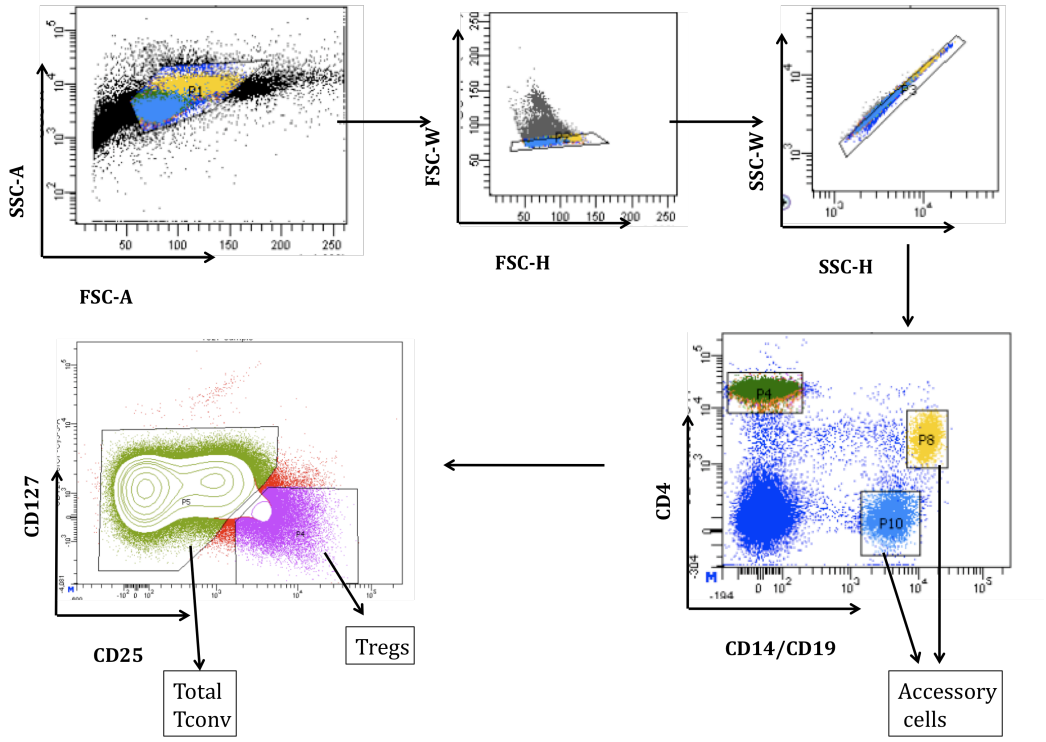

Figure 3.10 Gating strategy for the isolation of Tregs, total Tconv, B cells and monocytes for the Treg suppression assay used in the monozygotic twin study

Lymphocytes and monocytes were gated and doublets removed. After gating on CD4+ T cells, Tregs were isolated as before (Figure 3.1). Total CD4+ Tconv were isolated by selecting the CD4+ CD127hi CD25-/lo cells. CD14+ monocytes and CD19+ B cells were isolated together and were irradiated following collection.

bead:cell of 1:1, compared to 1:2.5 (Figure 3.11). Also when stimulating the cells with plate-bound anti-CD3 antibody, the higher the concentration, the higher the level of proliferation (Figure 3.11). Addition of soluble anti-CD28 antibody to the cultures enhanced proliferation further. The level of suppression produced at a Tconv:Treg ratio of 1:1 is shown in Figure 3.12. Similar results were found for the three other control donors examined (Figure 3.13). As observed earlier, stronger levels of stimulation resulted in lower levels of suppression.

3.2.6 Differences in suppression of Tconv proliferation between donors when stimulated with plate-bound anti-CD3 antibody

Although plate-bound anti-CD3-stimulated co-cultures in the absence of added soluble anti-CD28 antibody, showed higher levels of suppression than those with anti-CD28 antibody (Figure 3.12), the differences between donors were in most cases, larger when anti-CD28 was added (Figure 3.14). Therefore, based on these results and because previous studies examining Treg function in T1D had also used anti-CD28 antibody in their plate-bound anti-CD3 antibody-stimulated cultures (Lindley *et al.*, 2005, Lawson *et al.*, 2008, Brusko *et al.*, 2005) anti-CD28 antibody was added to these assays in the monozygotic twin study (Chapter Six).

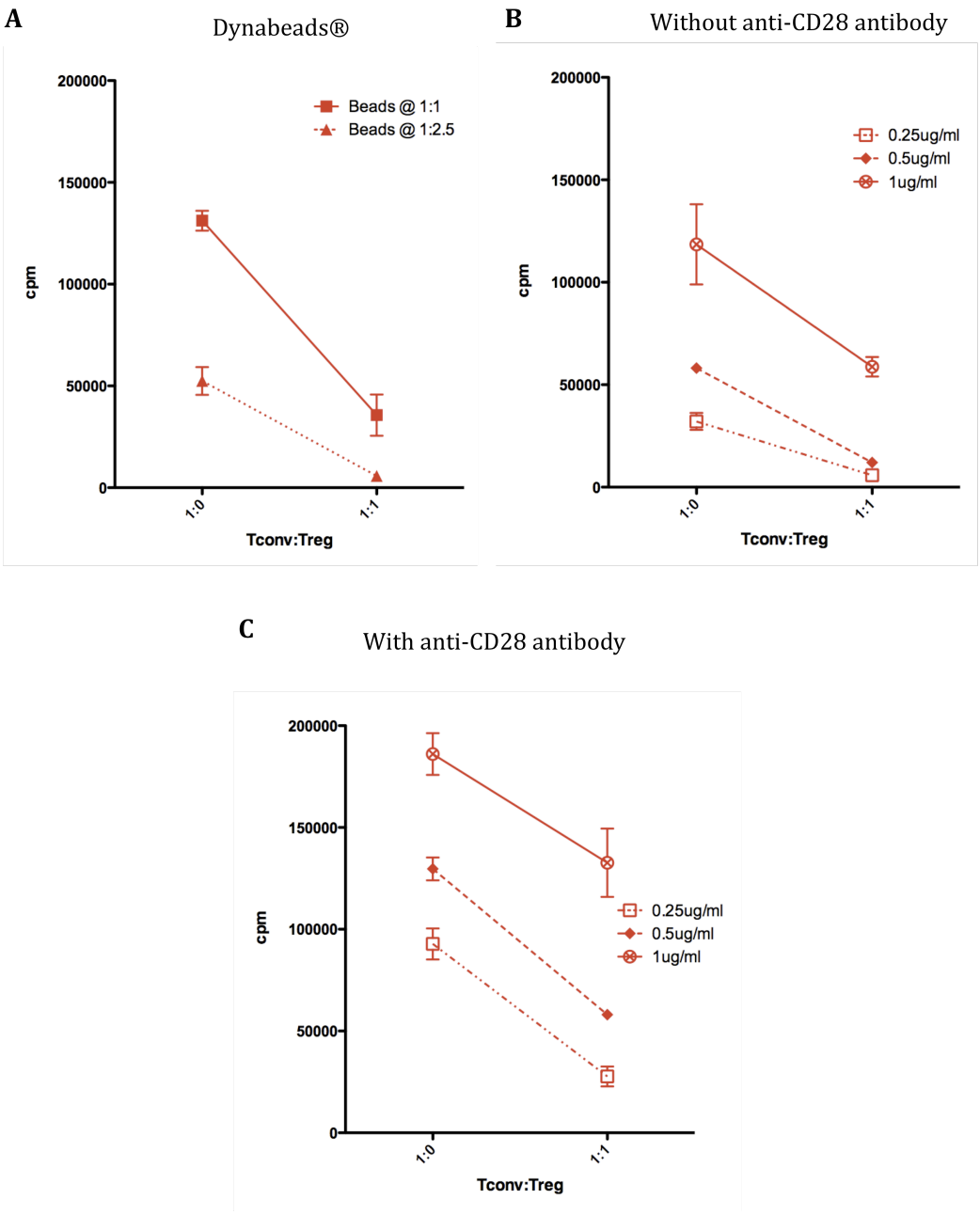

Figure 3.11 Comparison of proliferation in Dynabeads® and plate-bound anti-CD3 antibody stimulated co-cultures

Proliferation of total CD4+ Tconv from donor T027 in the absence or presence of autologous Tregs, when stimulated with bead:cell ratios of 1:1 ( ) and 1:2.5 ( ) (Figure A), or plate-bound anti-CD3 antibody at 0.25µg/ml ( ), 0.5µg/ml ( ) and 1µg/ml ( ) without soluble anti-CD28 antibody

(Figure B). Proliferation measured as mean cpm of 3[H]-thymidine across triplicate wells. Error bars show standard deviation. The data shown here are representative of all four donors tested.

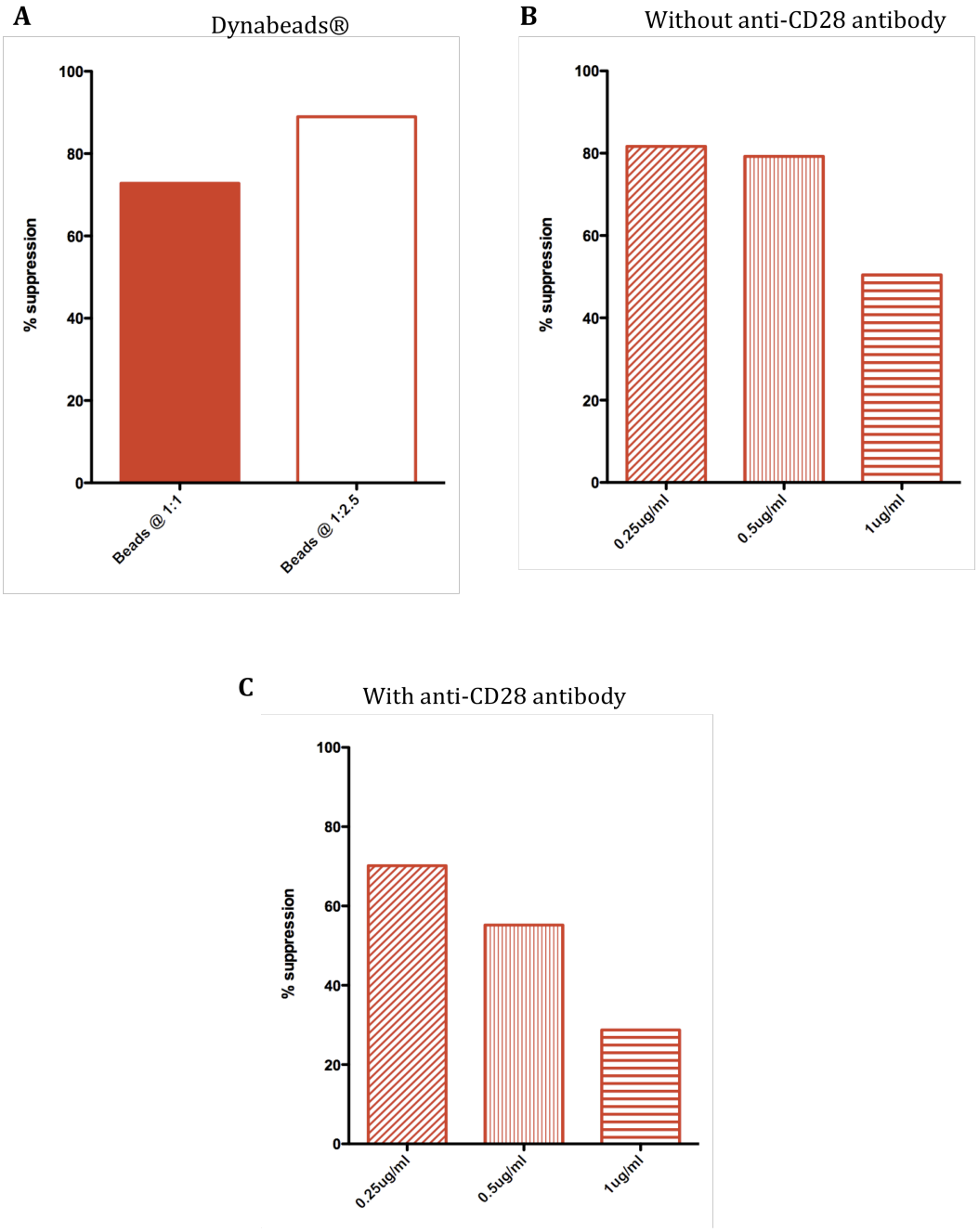

Figure 3.12 Comparison of suppression in Dynabeads® and plate-bound anti-CD3 antibody stimulated co-cultures

The mean level of suppression across triplicate wells of total Tconv proliferation from donor T027 (figure 3.11) in the presence of autologous

Tregs, when stimulated with bead:cell ratios of 1:1 (■) and 1:2.5 (□) (Figure A), or plate-bound anti-CD3 antibody at 0.25μg/ml (▨), 0.5μg/ml (▥) and 1μg/ml (▤) without soluble anti-CD28 antibody (Figure B) or with anti-CD28 antibody (Figure C). Suppression was calculated using the formula: % suppression= 100-(counts per minute (cpm) in the presence of Tregs ÷ cpm in the absence of Tregs) x 100). The data shown here are representative of all four donors tested.

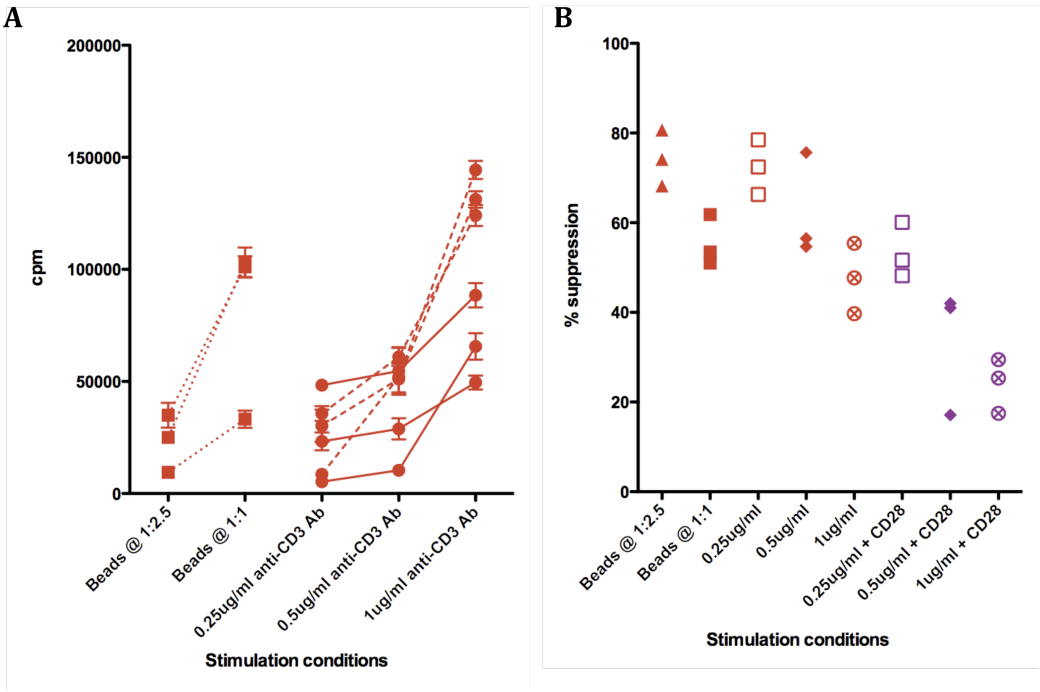

Figure 3.13 Comparison of suppression in Dynabeads® and plate-bound anti-CD3 antibody stimulated co-cultures for three other donors

Figure A shows the proliferation of CD4+ Tconv stimulated with beads or plate-bound anti-CD3 antibody for the three other donors. Error bars show standard deviation. Bead-stimulated co-cultures are shown by squares and dotted lines, whilst circles denote plate-bound anti-CD3 antibody-stimulated cultures. Solid lines show stimulation in the absence of anti-CD28 antibody and dashed lines show proliferation in the presence of anti-CD28 antibody. Figure B shows the mean levels of suppression calculated from Figure A using the formula: % suppression= 100-(counts per minute (cpm) in the presence of Tregs ÷ cpm in the absence of Tregs) x 100); bead:cell ratios of 1:2.5 (▲) and 1:1 (■) or plate-bound anti-CD3 antibody at 0.25µg/ml (□), 0.5µg/ml (◆) and 1µg/ml (⊗). Symbols shown in purple represent co-cultures stimulated with soluble anti-CD28 antibody.

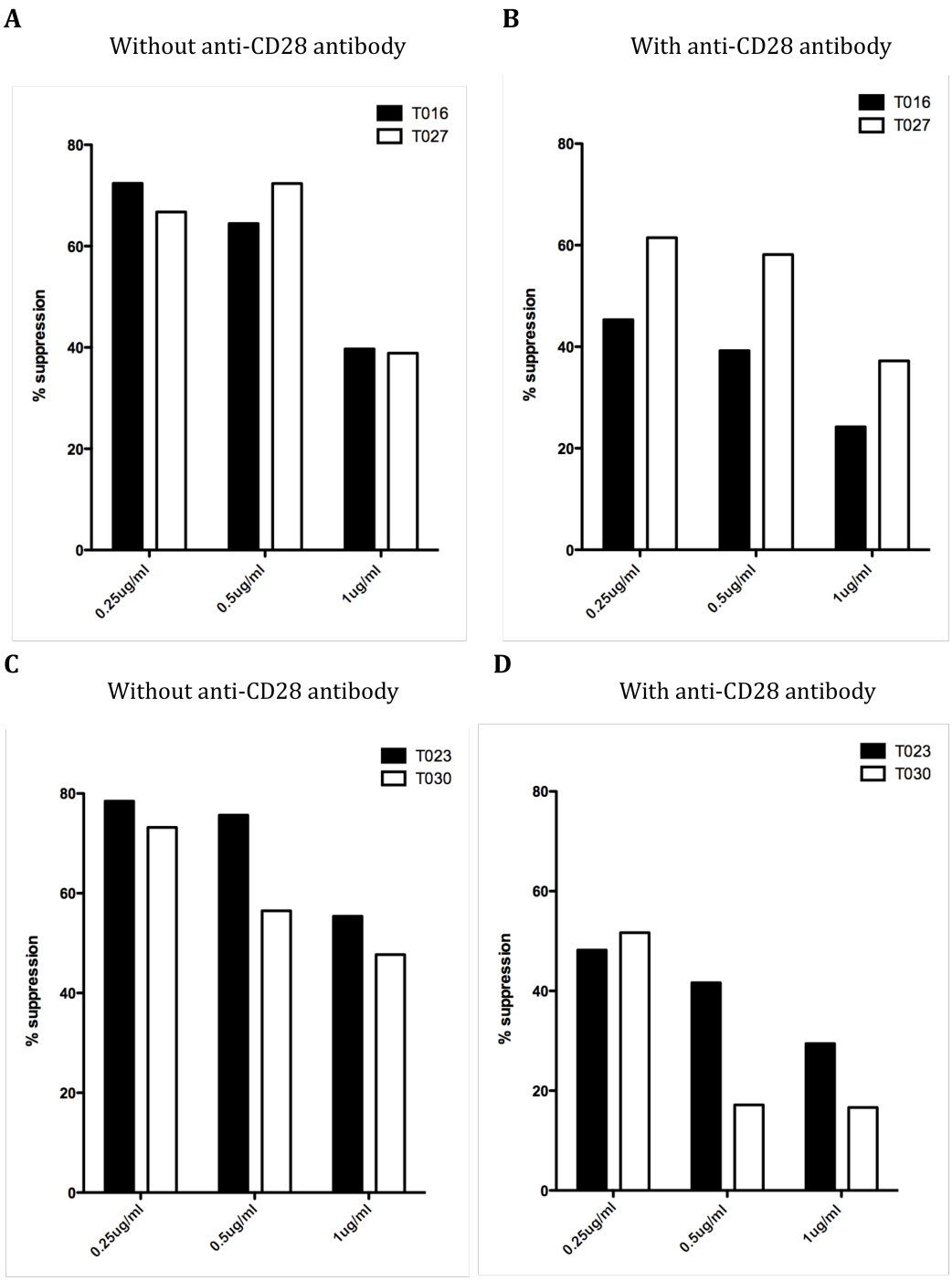

Figure 3.14 Difference in the level of suppression between donors in co-cultures stimulated with plate-bound anti-CD3 antibody is more pronounced with the addition of anti-CD28 antibody

The mean level of suppression of CD4+ Tconv proliferation in co-cultures

stimulated with 0.25μg/ml, 0.5μg/ml and 1μg/ml of anti-CD3 antibody, at a Tconv:Treg ratio of 1:1, is shown for two pairs. Suppression was calculated using the formula: % suppression= 100-(counts per minute (cpm) in the presence of Tregs ÷ cpm in the absence of Tregs) x 100). Figures A and B show one pair of donors, T016 (■) and T027 (□) whilst Figures C and D show the second pair of donors T023 (■) and T030 (□). Figures A and C show the level of suppression in co-cultures stimulated without anti-CD28 antibody, whilst Figures B and D show the level of suppression in co-cultures stimulated with 5μg/ml anti-CD28 antibody.

3.2.7. Reproducibility of the two-cell and three-cell suppression assays used to examine Treg function in pairs of monozygotic twins

The reproducibility of the two-cell suppression assay stimulated with Dynabeads® and the three-cell suppression assay stimulated with plate-bound anti-CD3 antibody-stimulated co-cultures with soluble anti-CD28 antibody was examined. Figure 3.15 compares suppression seen in response to all stimuli, in two pairs of donors, each assessed on two separate occasions. Although the level of suppression shows variation between assays, the pattern of the same donor showing more suppression than the other on both occasions occurs in nine out of ten cases.

As Tregs are a small population it was not possible to set up Treg suppression assays at all plate-bound anti-CD3 antibody concentrations tested. Therefore anti-CD3 antibody concentrations of 0.25μg/ml and 0.5μg/ml were selected for use. Although differences were demonstrated between control donors when cultures were stimulated with 1μg/ml (Figures 3.14 and 3.15) it may have been too strong a stimulus to use as part of an ideal dynamic range of stimuli.

3.2.8 Optimisation of the stimulation used in the Tr1 generation assay

As discussed in Section 3.1.2, to study the defective IL-2 signalling cascade seen in T1D, a Tr1-generation assay was used, in which the production of these cells was dependent upon IL-2. As one of the two anti-CD25

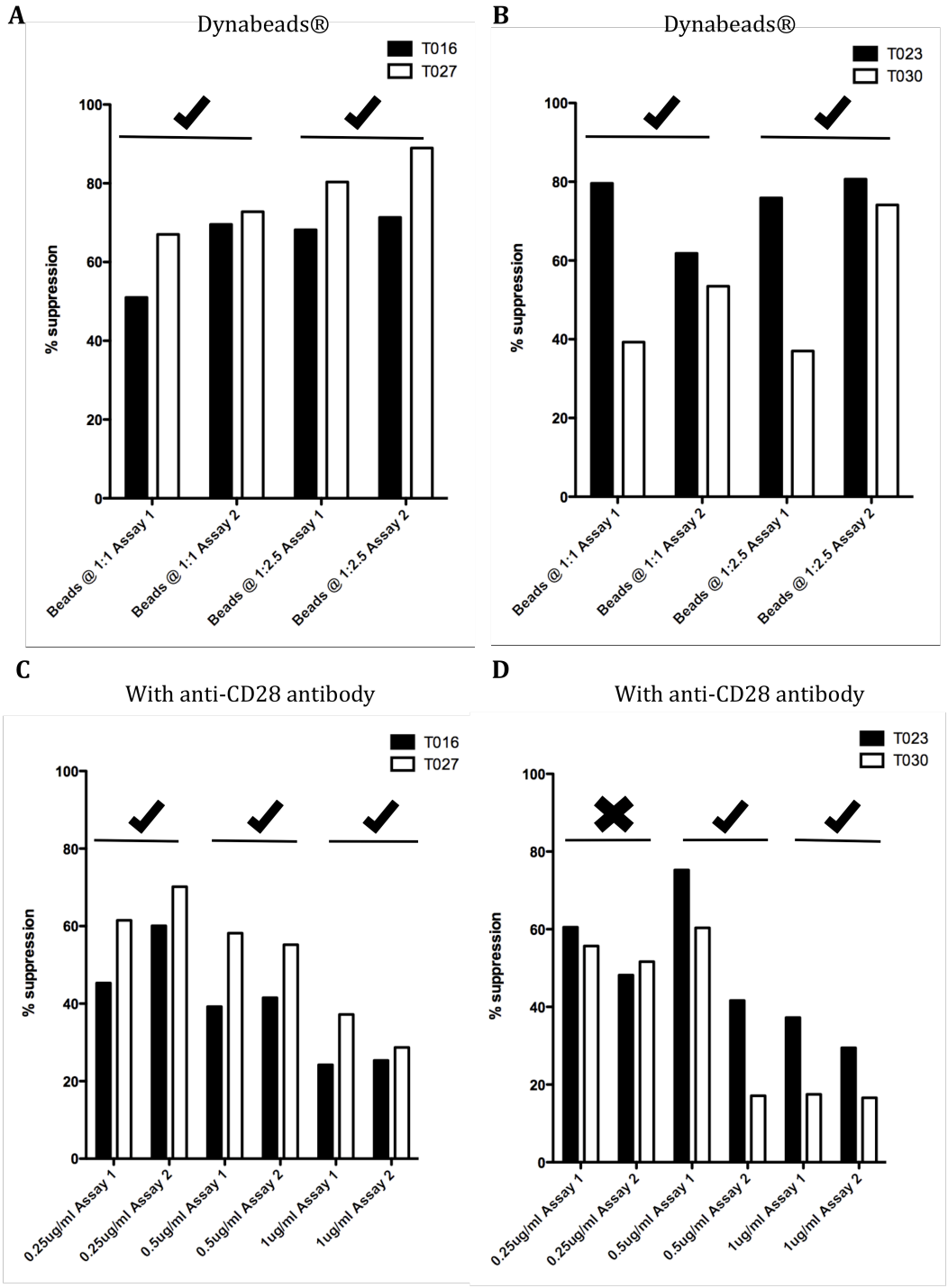

Figure 3.15 Reproducibility of suppression in suppression assays stimulated with Dynabeads® or plate-bound anti-CD3 antibody with soluble anti-CD28 antibody

Data show the mean level of suppression of CD4+ Tconv proliferation across

triplicate wells of co-cultures at a Tconv:Treg ratio of 1:1 stimulated with Dynabeads® (Figures A and B) or plate-bound anti-CD3 antibody with 5μg/ml anti-CD28 antibody (Figures C and D). Suppression was calculated using the formula: % suppression= 100-(counts per minute (cpm) in the presence of Tregs ÷ cpm in the absence of Tregs) x 100). Two pairs of donors are shown T016 (■) and T027 (□) in Figures A and C and T023 (■) and T030 (□) in Figures B and D. The suppression assays were conducted on two different occasions, following separate blood draws. Ticks are shown where the result was reproducible on both occasions a cross is given where reproducibility is not shown.

antibodies used in the isolation of Tregs and Tconv by FACS, functions as a CD25-blocking antibody (antibody clone 2A3) (Gronwall *et al.*, 2008) this antibody was omitted from the staining panel for the monozygotic twin study. This ensured generation of Tr1-like cells from sorted CD4+ Tconv was not impeded. As seen in Figure 3.16 omitting the 2A3 clone of anti-CD25 antibody did decrease the dynamic range of CD25 staining on cells, however, gating of Tregs was not impaired.

Although the clones of antibodies used in the Tr1-generation assay in this thesis were different to those in the study conducted by Kemper and colleagues (Cardone *et al.*, 2010) using them at the same concentrations (2.5μg/ml) was found to activate the Tconv sufficiently. As a comparison, Tconv were also stimulated with anti-CD3/anti-CD28 antibodies. IL-2 was tested at four concentrations (2, 10, 20 and 50 IU/ml) to examine whether this was a sufficient range to activate the cells. Production of IFNγ and IL-10 increased with increasing IL-2 concentration with higher levels produced in cultures stimulated with anti-CD3/anti-CD46 antibodies compared to those stimulated with anti-CD3/ anti-CD28 antibodies (Figure 3.17). In most donors, the highest levels of IL-10 were seen at 50 IU/ml although in some donors IL-10 production reached its peak at 20 IU/ml. Therefore this range of IL-2 concentrations tested was deemed suitable for this study.

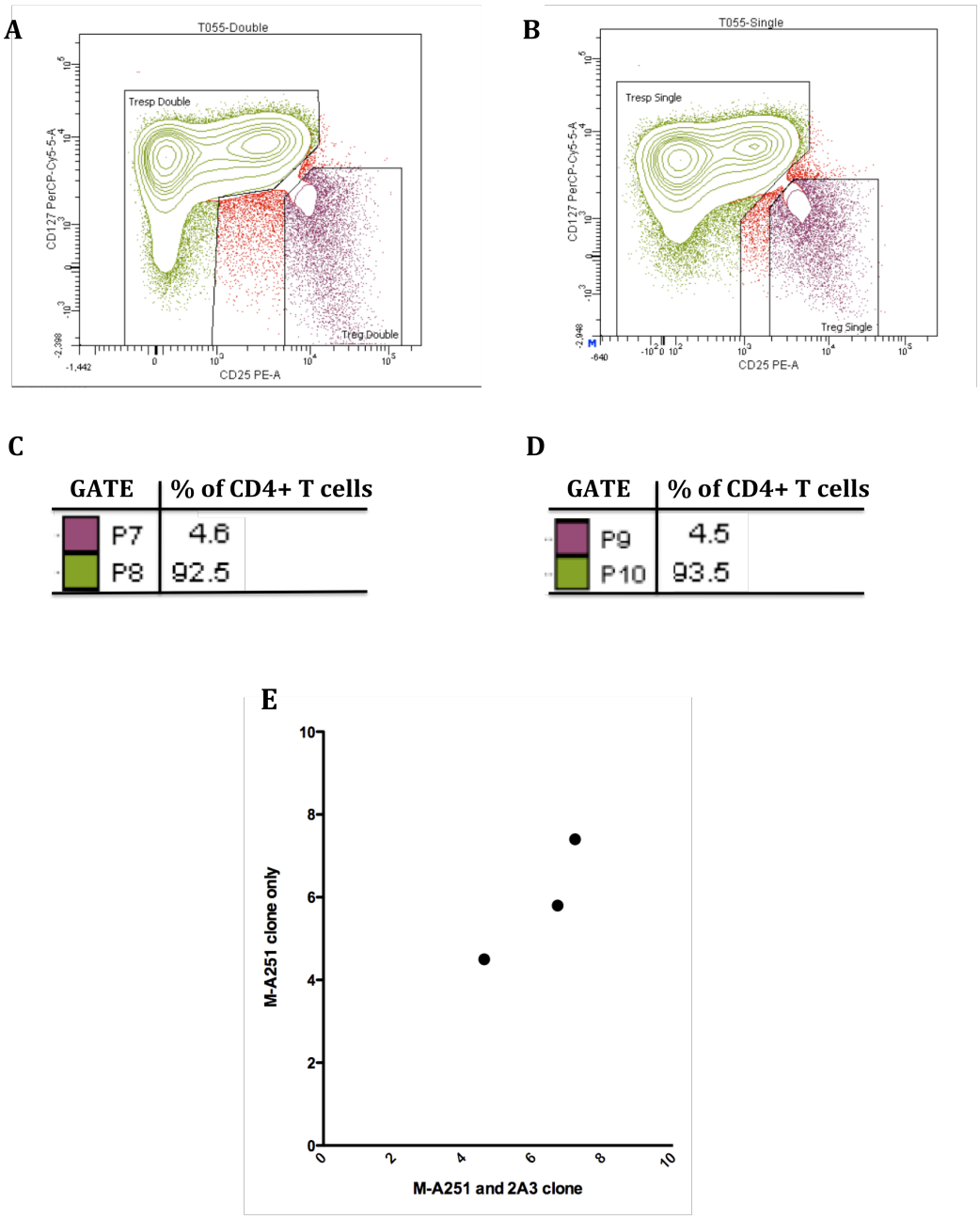

Figure 3.16 Use of one CD25 antibody instead of two does not compromise isolation of Tregs

Data compares the isolation of Tregs from donor T055, when the usual staining panel including the two different anti-CD25 antibodies, recognising different epitopes (clones M-A251 and 2A3) was used (Figures A and C), compared to when anti-CD25 antibody, clone 2A3, was omitted (Figures B and

D). The percentage of Tregs and CD4+ Tconv isolated by both staining panels is shown (Figure C and D). Comparison of the percentage of Tregs isolated when the 2A3 clone of antibody was omitted from the staining versus when it was included in the staining panel is shown for three individuals (including T055) (Figure E).

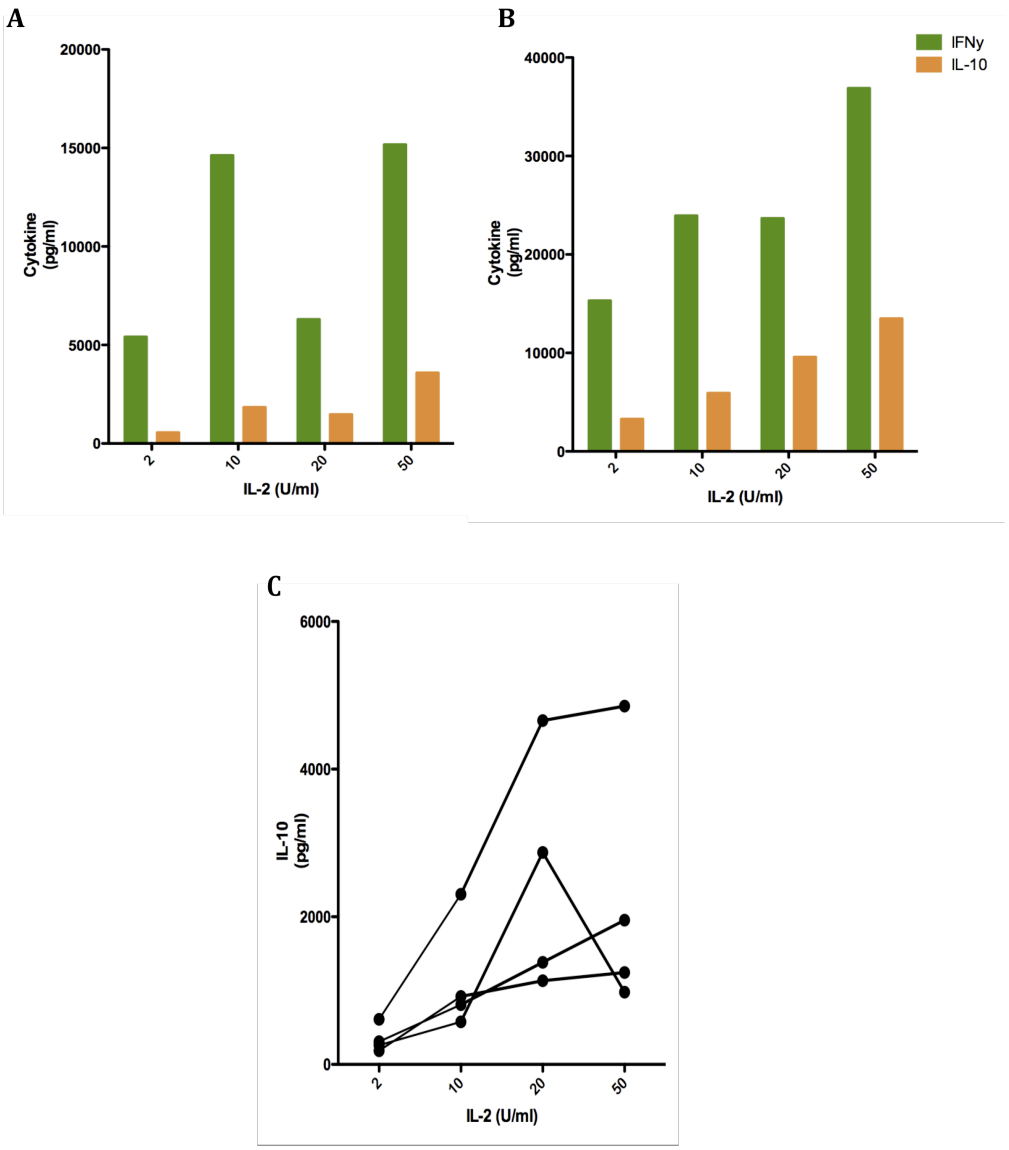

Figure 3.17 Production of cytokines by CD4+ in the Tr1 generation assay

Production of IFNγ and IL-10 (pg/ml) by CD4+ Tconv from one healthy control donor (T063) is shown in Figures A and B, following a three day stimulation with anti-CD3 antibody with either anti-CD28 antibody (Figure A) or anti-CD46 antibody (Figure B), when IL-2 was added at 2, 10, 20 or 50 IU/ml. Green bars show levels of IFNy production, whilst the orange bars show IL-10 production. The data shown in Figures A and B are representative of all nine

donors tested. The levels of IL-10 production at each IL-2 concentration is shown for four of the eight other donors tested (Figure C).

3.3 Discussion

The purpose of this Chapter was to fully optimise the assays implemented in this thesis. It was fundamental to the subsequent studies that the assays used were robust to ensure that the data obtained from each donor was reliable, of good quality and was known to be reproducible. A good dynamic range of stimuli was essential in both the Treg suppression assay and the Tr1-generation assay, to detect differences between the subjects tested.

3.3.1 Selection of a dynamic range of stimuli for the assays

In the subsequent sections of this thesis, Treg function was assessed by means of a suppression assay, which quantifies the inhibition of CD4+ Tconv proliferation, when in the presence of Tregs. Although this type of suppression assay was based upon those first described by Thornton and Shevach (Thornton and Shevach, 1998) and previous work conducted in this laboratory (Lindley *et al.*, 2005, Lawson *et al.*, 2008), the assay had to be optimised for its purpose in this thesis, as mentioned in Section 3.1.1. The stimuli strength used to activate the cells in this assay, was an important consideration, as has been highlighted by others. Baecher-Allan *et al*, demonstrated that increasing the concentration of the anti-CD3 antibody used to stimulate cultures, correlated with an increase in proliferation, but a concurrent decrease in suppression (Baecher-Allan *et al.*, 2001). Addition of anti-CD28 antibody to these cultures enhanced this effect. As these authors used CD4+ CD25- T cells, this would be expected as this population often

characterises the naïve Tconv population, which require both stimulation through the TCR and from CD28-CD80/86 ligation to become activated (Croft *et al.*, 1994). The reason for lower levels of suppression at higher stimuli strengths may be because self-antigens stimulate autoreactive T cells weakly and inhibition of these responses is desirable, whereas stimulation by foreign antigens is stronger (Baecher-Allan and Hafler, 2006, Walker and Abbas, 2002, Bouneaud *et al.*, 2000). Also, as explained in Chapter One, Section 1.7, infections cause an up-regulation of CD80/86 on APC, thus explaining the effect of anti-CD28 antibody on decreasing suppression (Walker and Abbas, 2002).

A good dynamic range of stimuli strengths was required to detect variations between patients and controls. In a study examining Treg function in individuals with RRMS, the authors found that when using low-dose plate-bound anti-CD3 antibody (0.1 and 0.5μg/ml) the patients showed significantly lower levels of suppression versus controls. However, when the anti-CD3 antibody was increased to 2.5μg/ml, comparable poor levels of suppression were seen in both sets of donors (Viglietta *et al.*, 2004). Similar results have also been found when assessing Treg function in T1D. In a study measuring Treg suppression in newly diagnosed patients, these T1D donors had significantly less suppression versus controls, when cells were stimulated with 5μg/ml of anti-CD3 antibody. However, when this stimulus was increased to 10μg/ml, both T1D and non-T1D donors showed equally

low levels of suppression (Lindley *et al.*, 2005).

As well as using too high a dose, stimulating the cells too weakly also hinders the identification of differences in suppression. Lawson *et al* (2008) demonstrated that decreasing the concentration of anti-CD3 antibody from 1μg/ml to 0.3μg/ml, resulted in comparable, fairly high levels of suppression between long-standing diabetics and non-diabetics. Thus, at low levels of stimulus, both donor sets display low levels of proliferation and similarly high levels of suppression, whereas at high levels of stimuli, proliferation is great and suppression is low, yet comparable. Therefore, the stimuli selected needs to be within an intermediate range, allowing any differences to be revealed (Figure 3.18).

The use of an inappropriate range of stimuli strengths may explain why one paper demonstrated no difference in suppression between diabetic donors and controls. The stimuli used were either very low (0.01 and 0.5μg/ml of plate- bound anti-CD3 antibody) or very high (2.5μg/ml of plate-bound or soluble anti-CD3 antibody with 2.5μg/ml anti-CD28 antibody) (Putnam *et al.*, 2005). Had they included intermediate concentrations in this range of stimuli, the authors may have seen a difference.

In this thesis, Dynabeads® at ratios of 1:1 and 1:2.5 were selected over a ratio of 1:5, which resulted in very low levels of proliferation. For plate-

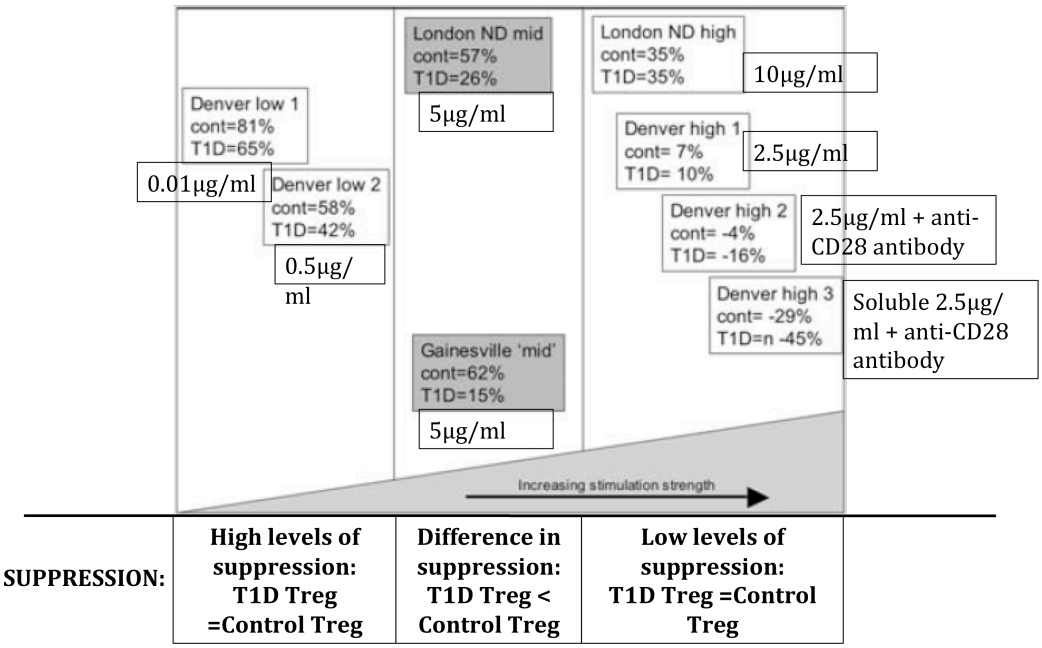

Figure 3.18 Levels of suppression between controls and T1D donors at different stimuli strengths

The diagram above illustrates the results from three different studies examining Treg function in T1D, the London study (Lindley et al., 2005) examining ROT1D, the Denver study (Putnam et al., 2005) examining LST1D and the Gainesville study (Brusko et al., 2005) studying both ROT1D and LST1D. Percentage suppression in all studies was calculated from the mean level of CD4+ CD25- Tconv suppression using the formula: % suppression= 100-(counts per minute (cpm) in the presence of Tregs ÷ cpm in the absence of Tregs) x 100). No difference in Treg suppression was detected between T1D and controls at high or low signal strengths, only at middling concentrations. The anti-CD3 antibody concentrations that were used are shown. It should be noted that differences in assay technique might also influence Treg suppression. Data reproduced from Tree et al., 2006.

bound concentrations, 0.25 and 0.5μg/ml of anti-CD3 antibody were selected. 1μg/ml resulted in high levels of proliferation, but lower suppression as expected and therefore, may not have been appropriate to use. Anti-CD28 antibody was added to co-cultures, despite lower levels of suppression, as these cultures showed higher levels of reproducibility, bigger differences between donors and also were implemented in other studies examining Treg function in T1D (Lawson *et al.*, 2008, Lindley *et al.*, 2005, Brusko *et al.*, 2005).

3.3.2 Optimisation of Tr1 generation assays

As already mentioned in Section 3.1.2, the Tr1-generation assay was based upon those conducted by Cardone *et al.* although optimisation was required (Cardone *et al.*, 2010). The omission of the 2A3 clone of anti-CD25 antibody from the FACS staining panel did not impede the gating of Tregs, with little difference to the yield obtained. A range of IL-2 concentrations were tested (2, 10, 20 and 50 IU/ml) and showed an IL-2-dependent increase in IL-10 production by the Tr1-like cells, especially when stimulated with anti-CD3/46 antibodies. Therefore these concentrations were used as an ideal dynamic range of stimuli, to test not only the level of IL-10 produced by the CD4+ Tconv between pairs of monozygotic twins but also the ability of the cells to switch from T_H1-like cells to Tr1-cells as the IL-2 concentration administered increased (see Chapter One, Section 1.5).

Chapter Four: Examination of the implications of the *IL-2RA* SNP, rs2104286, on regulatory T cell function

4.1 Introduction

As discussed in Chapter One, Section 1.13.4, several SNPs in the IL-2RA gene coding for CD25, are associated with T1D (Lowe *et al.*, 2007, Maier *et al.*, 2009, Dendrou *et al.*, 2009). Genotype-phenotype studies conducted by our collaborators at the University of Cambridge, demonstrated that two of these SNPs are associated with the phenotype expressed by CD4+ Tconv (Dendrou *et al.*, 2009). A protective allele at SNP.495 is associated with a significantly higher level of CD25 expression by memory Tconv and IL-2 production by these cells when stimulated, whilst a protective allele at SNP.286 is associated with significantly lower percentages of CD25+ naive Tconv (Dendrou *et al.*, 2009). In both cases the protective allele is the minor allele (Maier *et al.*, 2009, Dendrou *et al.*, 2009). Although neither of these SNPs have been associated with changes in the Treg phenotype (Dendrou *et al.*, 2009), it is possible the differences in Tconv phenotypes may influence their sensitivity to Treg function.

It is not yet apparent what population CD4+ CD25+ naïve Tconv represent, but it is possible that these cells, particularly from susceptible individuals, could be in a state of early activation. Indeed, work by Todd and colleagues demonstrated that following stimulation, a significantly higher percentage

of CD25+ naïve Tconv from donors homozygous for the susceptible allele at SNP.286 expressed the early activation marker CD69, compared to those homozygous for the protective allele (Dendrou *et al.*, 2009). Also, studies have shown that CD25 and CD69 begin to be up-regulated within the first few hours of activation *in* vitro (Poulton *et al.*, 1988, Testi *et al.*, 1989) whereas the down regulation of CD45RA on naïve Tconv *in vitro* is a gradual process commencing once the cells begin to divide (Ma *et al.*, 2004). Furthermore, recent work has shown CD25+ naïve Tconv to have higher levels of both CD25 and CD122 than CD25- naïve Tconv, thus making them more responsive to IL-2 (Pekalski *et al.*, 2013).

Alternatively, it could also be the case that CD25+ naïve Tconv represent a population of cells whose activation has been aborted before it could reach completion. Although the percentage of CD25+ naïve Tconv increases with age, the percentage of these cells within each donor remains constant when assessed over a period of three months (personal communication-(Pekalski, 2012)). This suggests that rather than being cells undergoing activation, these could actually be cells halted in a transitory phase from naïve Tconv to memory Tconv. Although, this may seem more fitting of a tolerogenic mechanism with autoreactive Tconv prevented from becoming activated, it may be that by inhibiting larger numbers of cells from differentiating into memory Tconv, the levels of IL-2 produced would be considerably lower, thus affecting the Treg population, which require this cytokine for maintenance (Taams *et al.*, 2001, Zorn *et al.*, 2006).

From their examination of *IL-2RA* haplotypes, our collaborators demonstrated that the protective allele at SNP.286 is present on two haplotypes, the Protective P1 haplotype and the Protective P3 haplotype, whereas the protective allele at SNP.495 is only present on the Protective P1 haplotype (Dendrou *et al.*, 2009). (It should be noted that the Protective P2 haplotype with a protective allele at another T1D-associated *IL-2RA* SNP; SNP.656, is not associated with changes in either Treg or CD4+ Tconv phenotypes (Dendrou *et al.*, 2009).) The Fully Susceptible haplotype has susceptible alleles at SNP.495 (and SNP.656) and SNP.286 (Dendrou *et al.*, 2009). Figure 1.6 summarises the presence of protective or susceptible alleles at the SNPs comprising these haplotypes.

Interestingly, although SNP.286 is associated with T1D (Lowe *et al.*, 2007, Maier *et al.*, 2009), only the Protective P1 haplotype is associated with this disease as the Protective P3 haplotype occurs with equal frequency in both T1D and controls (Dendrou *et al.*, 2009). It is not clear whether the reason SNP.286 is associated with T1D is because of its high linkage disequilibrium with SNP.495, hence why it is also present on the Protective P1 haplotype or whether this SNP is truly linked with the disease.

The aim of this chapter was to examine the hypothesis that SNP.286 influences the ability of CD4+ naïve Tconv to maintain Tregs, thus affecting their suppression. Also this study was designed as a proof- of-principle that

such a genotype-immunophenotype could be conducted. Prior to conducting this study, power calculations were calculated to determine the minimum number of subjects required. Previous work in this laboratory showed that the average level of Tconv suppression by Tregs from LST1D was 38% and that from controls was 62%, together with a S.D. of 19% (Lawson, 2008). Therefore the power calculation showed that if a similar study was conducted, to be 95% confident of no Type 1 Error (a false positive, see Chapter Six, Section 6.3.1) and to be 80% certain of being able to detect a difference, a minimum of ten donors was required (Brant, 2013). However, this simple calculation did not take into account factors such as all donors being non-diabetic and the genotype of these individuals. More complex power calculations were conducted by our collaborators which demonstrated that a minimum of forty donors was actually required (personal communication-(Tree, 2013). It was not possible to obtain this number of donors from the donor cohort used. Also as this study was conducted as a proof-of-principle only eight donors were used.

Ideally, non-diabetic donors homozygous for the Protective P3 haplotype would have been selected for this study, however due to the small number of these individuals in the donor cohort used (our collaborators have reported that only 15% of controls from their vast cohort of donors; the Cambridge BioResource (see Chapter Five, Section 5.1.2) were Protective P3 homozygous (Dendrou *et al.*, 2009)) heterozygous donors, expressing one protective allele at SNP.286 on one haplotype and one susceptible allele on

the other, were recruited. For the purposes of this study, a pair-wise analysis was conducted with each pair consisting of one protective donor (Protective P3 haplotype heterozygous) and one susceptible donor (Fully Susceptible haplotype homozygous). The cells of interest from each of these donors were isolated by FACS (as described in Chapter Two, Section 2.8.4 and Chapter Three Section 3.2.1). Proliferation and suppression of the Tconv populations were measured by a two-cell Treg suppression assay activated by Dynabeads® at bead:cell ratios of 1:1 and 1:2.5 (as described in Chapter Two, Section 2.8.8 and Chapter Three, Section 3.2.2). In addition to co-culturing the Tconv with autologous Tregs, two-cell Treg suppression assays were also set-up using Standard Tregs to observe differences in the resistance of Tconv to suppression (see Chapter Three, Sections 3.2.3 and 3.2.4). Additional work was conducted by Ms Garima Garg to examine differences in the IL-2 signalling cascade, by measuring FOXP3 maintenance and apoptosis in cells immediately after sorting and following culture for forty-eight hours in the absence or presence of exogenous IL-2 (see Chapter Two, Sections 2.8.5 and 2.8.6). This work is presented here, for the purpose of completeness, with her kind permission. Statistical analyses were performed as described in Chapter Two, Section 2.8.11 and are given in each figure. A summary of all procedures conducted on the samples is shown in Figure 4.1, indicating which individual performed each aspect of the work.

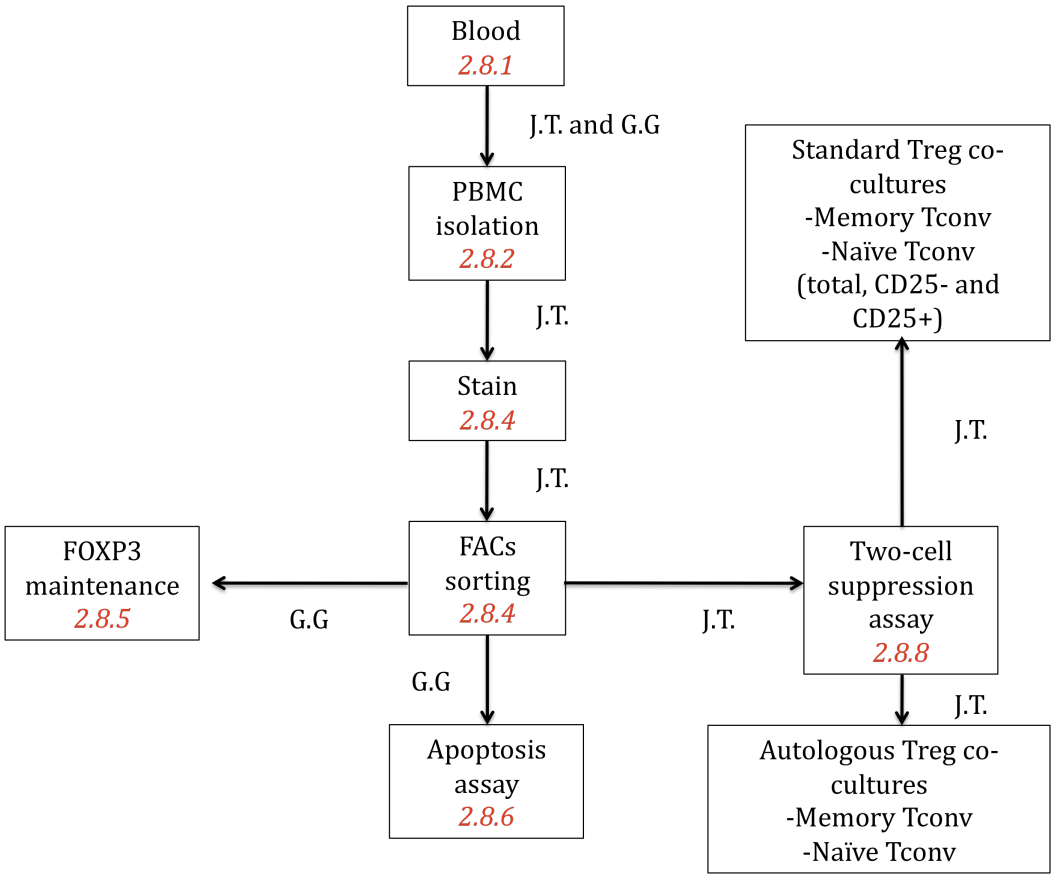

Figure 4.1 Flow chart summarising the procedures conducted on each sample

The above flow chart demonstrates the order and number of all procedures conducted on each pair of samples. The initials of the laboratory worker involved at each stage are given (J.T. Ms Jennifer Tyler, G.G Ms Garima Garg). The red italics show the section in Chapter Two giving full details of protocols used.

4.2 Results

4.2.1 Donor selection

To investigate the possible implications of SNP.286 on Treg function protected and susceptible donors were recruited from the King's College London cohort of donors and were studied in a pair-wise fashion (Table 4.1). To avoid any confounding factors of the disease, non-diabetic donors were selected. The immunophenotype associated with the allele at SNP.286 is the percentage of CD25+ naïve Tconv (Dendrou *et al.*, 2009), however, this is not influenced by genotype alone and further investigation has shown that males show significantly lower percentages of these cells than females (Dendrou *et al.*, 2009) and the percentage of these cells also shows a positive correlation with increasing age (Dendrou *et al.*, 2009). Therefore all donors were gender- and age-matched (Protected donors: mean age ± S.D.: 35.38 ± 9.30 and susceptible donors mean age ± S.D.: 31.38 ± 6.05). It should be noted that the utmost importance was placed upon each pair of samples being treated, as far as was possible, exactly the same way in a bid to reduce bias generated by day-to-day variations. Prospectively it may appear that some donors would have been better matched with others, however unavoidable factors, such as one donor being suddenly unavailable when they were due to be bled meant that another donor from the cohort deemed to be the most suitable match was recruited in their place. The protected donor from pair five (marked by the red squares in Table 4.1) had the Protective P1 haplotype, which has a protective allele at SNP.286 and

Pair no.	Symbol	Protective Haplotype		Susceptible Haplotype	
		Gender	Age	Gender	Age
1		M	26	M	28
2		M	45	M	35
3		M	35	M	43
4		F	42	F	34
5		F	35	F	33
6		F	25	F	26
7		M	49	M	36
8		M	26	M	26

Table 4.1 Details of the donors recruited for the study examining the implications of the P3 haplotype on Treg function

M=Male, F=Female.

The red squares highlight the protected donor with the Protected P1 haplotype instead of the Protected P3 haplotype.

also SNP. 495 (see Chapter One, Section 1.13.4 and Section 4.1) (Dendrou *et al.*, 2009). Therefore, data on the memory Tconv from this donor were excluded from all subsequent analyses.

4.2.2 Isolation of the cells

As described in Chapter Two, Section 2.8.4 and Chapter Three, Section 3.2.1, total CD4+ CD25hi CD127lo Tregs, total CD4+ memory Tconv, total CD4+ naïve Tconv and the CD25- and CD25+ subpopulations of CD4+ naïve Tconv were isolated from PBMC from each donor by FACS (Figure 4.2). Memory Tconv were isolated according to the absence of CD45RA, whilst naïve Tconv were isolated according to its presence. After the desired number of naïve Tconv was collected, CD25- and CD25+ naïve Tconv were then isolated.

4.2.3 Analysis of the CD4+ naïve Tconv population

As the protective allele at SNP.286 is associated with a decrease in the percentage of CD4+ CD25+ naïve Tconv (Dendrou et al., 2009) the percentages of total CD4+ naïve Tconv and CD25+ naïve Tconv were analysed between the protected and susceptible donor groups (Figure 4.3). No significant differences were seen between the two sets of donors regarding the percentage of either of these two cell populations. No significant difference in the level of CD25 expression on memory or naïve

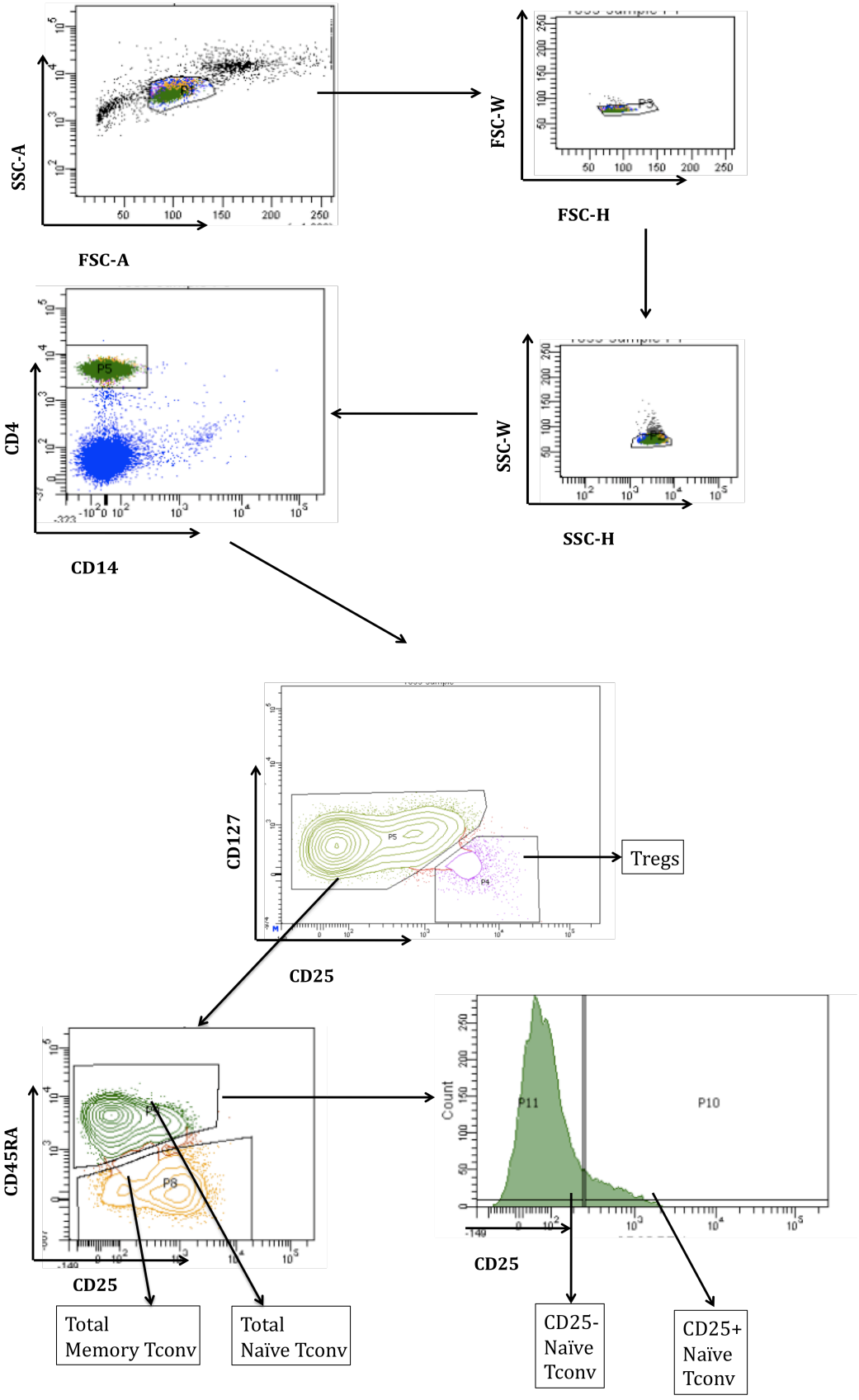

Figure 4.2 Gating strategy for the isolation of Tregs, total memory, total

naïve and naïve CD25-/+ Tconv

The gating used to isolate cells for the IL-2RA SNP.286 genotype-immunophenotype study is shown. Firstly, lymphocytes were gated and doublets removed. After gating on CD4+ T cells, the Tconv were selected by high expression of CD127 and negative to intermediate expression of CD25. Tregs were isolated according to high expression of CD25 correlating with low levels of CD127. Memory Tconv were isolated according to the absence of CD45RA, whilst naïve Tconv were isolated according to its presence. After the required number of naïve Tconv was collected, the remaining naïve Tconv population was divided according to the absence or presence of CD25.

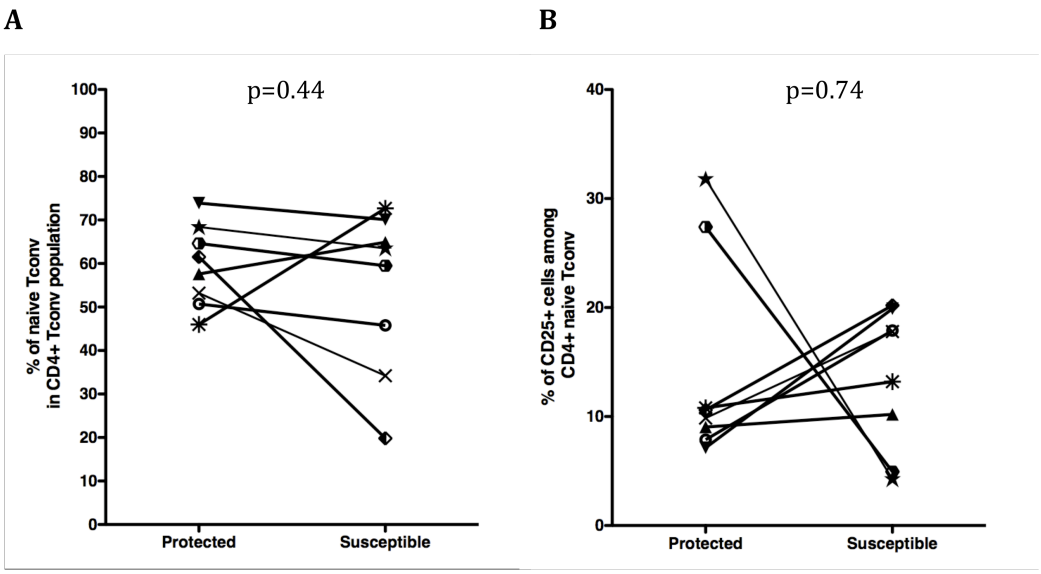

Figure 4.3 Percentages of total CD4+ naïve and CD25+ naïve Tconv

Percentages of the total population and CD25+ subpopulation of CD4+ naïve Tconv were compared between the protected and susceptible donor groups. Figure A shows the percentages of total CD4+ naïve Tconv and Figure B shows the percentages of CD4+ CD25+ naïve Tconv. Data in Figure A was analysed by a two-tailed paired t test and data in Figure B was analysed by a two-tailed Wilcoxon matched-pairs signed rank test.

Tconv were seen between the donor groups either (data not shown).

4.2.4 Examination of total and subpopulations of Tregs

Our collaborators have shown that SNP.286 is not associated with any difference in the level of CD25 expression on total CD4+ FOXP3+ Tregs (Dendrou *et al.*, 2009). However they did not look at actual percentages of Tregs. The percentage of total Tregs was measured for both groups of donors but no differences between the two donor sets was seen (Figure 4.4). As discussed in Chapter One, Section 1.3 Tregs are not a homogenous population and work by Sakaguchi and colleagues demonstrated the presence of three subpopulations identified by differences in the expression of either CD25 or FOXP3 against CD45RA; rTregs, mTregs and aTregs (Miyara *et al.*, 2009). FOXP3 was not used during the sort as these cells needed to be viable for the following assays, therefore the markers CD25 and CD45RA were used. These cells were gated as shown in Figure 4.4B. Analysis of each of these subpopulations revealed no significant difference in the percentages of rTreg or mTreg between the two groups of donors (Figure 4.4 C and D). However, protected donors had significantly more aTregs than susceptible donors (Figure 4.4 E). The level of CD25 expression on the surface of these cells was also analysed, but there were no significant differences between the two donor sets (data not shown).

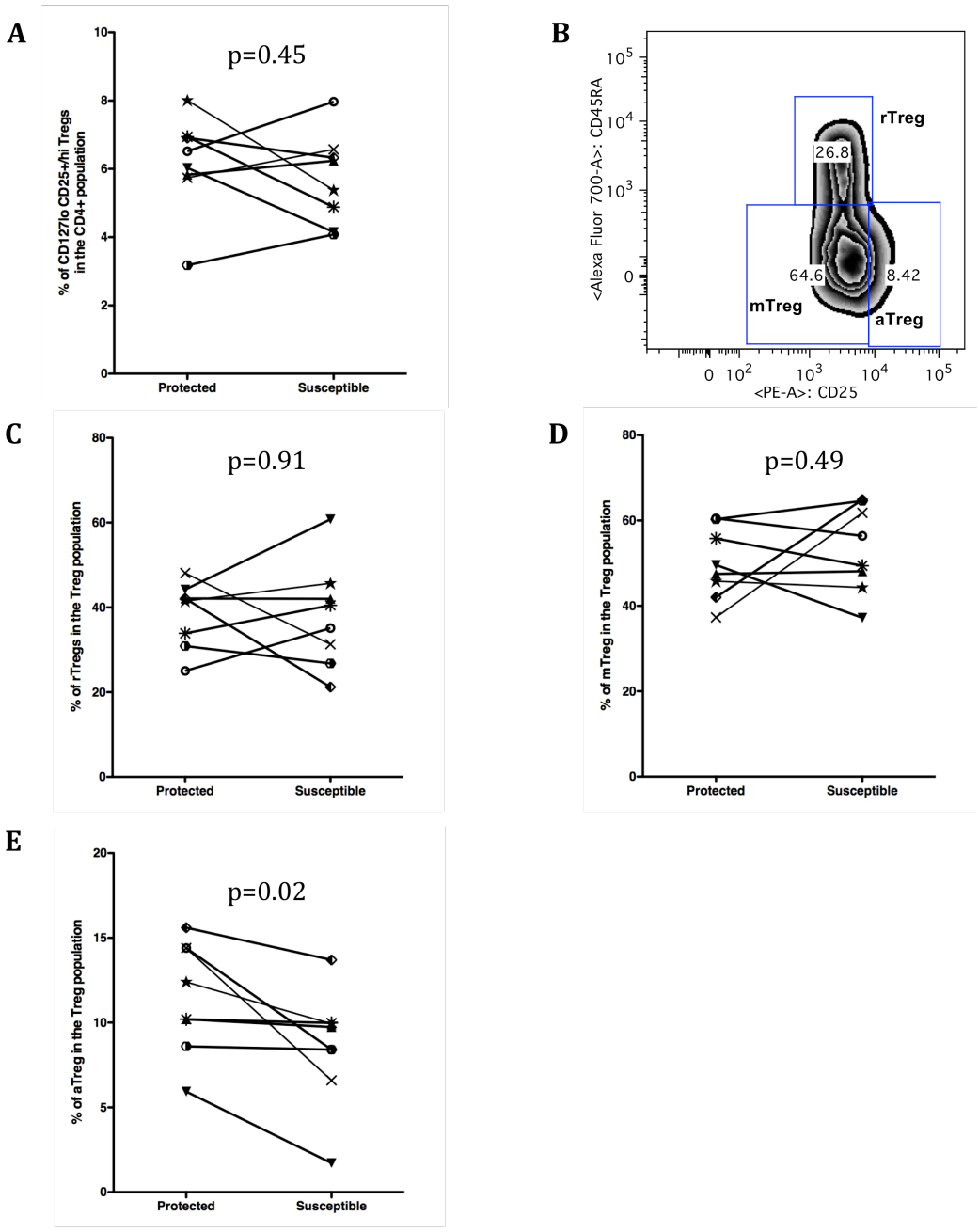

Figure 4.4 Percentages of total and subpopulations of Tregs

Figure A shows the percentage of total CD4+ CD25+ CD127lo Tregs. Figure B shows the gating strategy used to select the three Treg subpopulations. The percentage of rTregs (Figure C), mTregs (Figure D) aTregs (Figure E) in the CD4+ T cell population is also shown. Data was analysed by a two-tailed paired t test.

4.2.5 Proliferation of the different Tconv populations

Two-cell Treg suppression assays were stimulated with Dynabeads® at bead:cell ratios of 1:1 and 1:2.5. The proliferation of the different Tconv populations was compared to see if there was any difference in the way in which the cells responded to the stimuli (Figures 4.5 and 4.6). Unfortunately due to a technical error, no naïve Tconv (total and both CD25 subpopulations) were sorted from pair one and there was not enough of the CD25 subpopulations sorted from pair two to set up the suppression assays for both donors with these cells. Therefore, only seven pairs were analysed for naïve Tconv and six pairs for the CD25 subpopulations. Also memory Tconv from pair five was omitted as the protected donor has the Protective P1 haplotype (see Section 4.2.1). Statistical analysis showed that when stimulated at a bead:cell ratio of 1:1, protected donors had a significantly higher level of naïve Tconv proliferation than susceptible donors. Interestingly, at this stimuli strength, proliferation of CD25 negative naïve Tconv is close to significance ($p=0.06$) with protected donors showing higher levels of proliferation than susceptible donors. No significant differences between donor sets were seen at a lower stimulus of 1:2.5 (Figure 4.6).

4.2.6 Assessment of the correlation between the percentage of CD25+ naïve Tconv and proliferation

Recent observations by Todd and colleagues demonstrate that CD25+ naïve

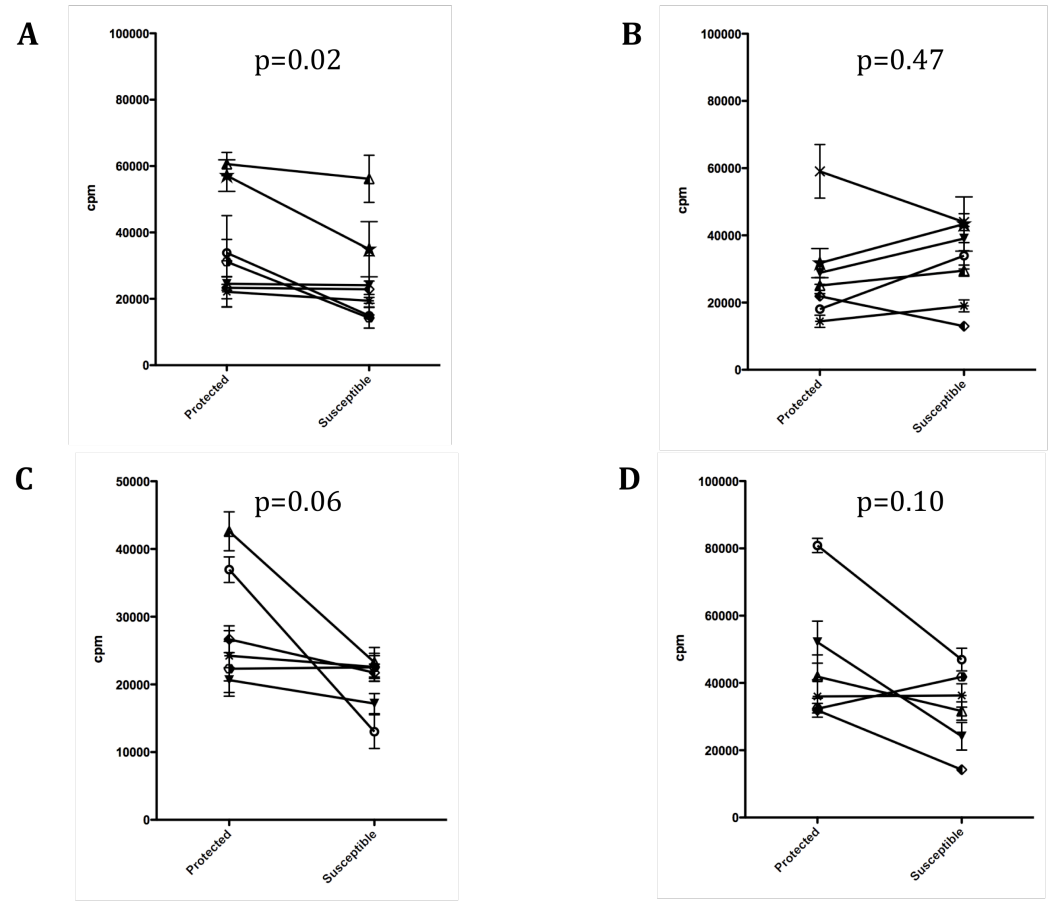

Figure 4.5 Proliferation of CD4+ Tconv subpopulations when stimulated with a bead:cell ratio of 1:1

Proliferation of total naïve Tconv (Figure A) total memory Tconv (Figure B), CD25- naïve Tconv (Figure C) and CD25+ naïve Tconv (Figure D) when stimulated with a bead:cell ratio of 1:1. Proliferation is shown as mean cpm of 3[H]-thymidine in triplicate wells and error bars show standard deviation. Data in Figures B and D was analysed by a two-tailed paired t test and data in Figures A and C was analysed by a two-tailed Wilcoxon matched-pairs signed rank test.

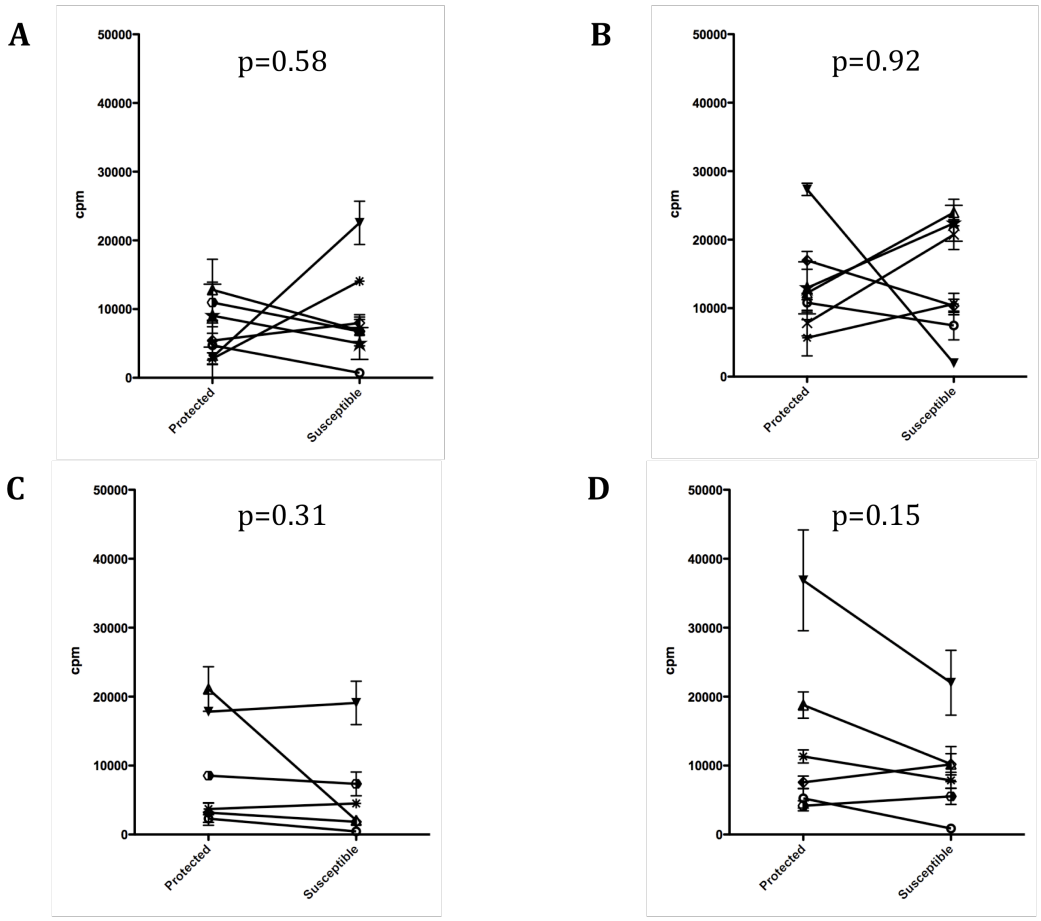

Figure 4.6 Proliferation of CD4+ Tconv subpopulations when stimulated with a bead:cell ratio of 1:2.5

Proliferation of total naïve Tconv (Figure A) total memory Tconv (Figure B), CD25- naïve Tconv (Figure C) and CD25+ naïve Tconv (Figure D) when stimulated with a bead:cell ratio of 1:2.5. Proliferation is shown as mean cpm of 3[H]-thymidine in triplicate wells and error bars show standard deviation. Data was analysed by a two-tailed paired t test.

Tconv are more easily activated than CD25- naïve Tconv (Pekalski *et al.*, 2013). Therefore, the level of proliferation under both Dynabeads® stimuli (bead:cell ratios of 1:1 and 1:2.5) was investigated (Figure 4.7). At both signal strengths, the CD25+ naïve Tconv proliferation was significantly higher than CD25- naïve Tconv. This difference is even more pronounced at the higher signal strength of 1:1. As the CD25+ naïve Tconv population proliferates more than the CD25- population, the percentage of these cells in the naïve population could possibly influence the proliferation of total naïve Tconv. However, correlations between the percentage of these cells and proliferation of naïve Tconv showed no significance at either signal strength (Figure 4.7 C and D).

4.2.7 FOXP3 maintenance in Tregs

IL-2 is required to maintain FOXP3 (Zorn *et al.*, 2006) and the signalling cascade of this cytokine has been reported to be defective in T1D (Long *et al.*, 2010, Long *et al.*, 2011) (Chapter One, Section 1.14.5). Although no difference in Treg CD25 expression was seen between groups of donors, any possible effects this SNP may have on the IL-2 signalling pathway were examined by measuring the maintenance of FOXP3 in Tregs immediately after sorting and after a 48-hour incubation in the absence of IL-2 or with 2 or 20 IU/ml of exogenous IL-2. This work was conducted by Ms Garima Garg as described in Chapter Two, Section 2.8.5. No significant differences in the level of FOXP3 expression at any of these conditions were seen

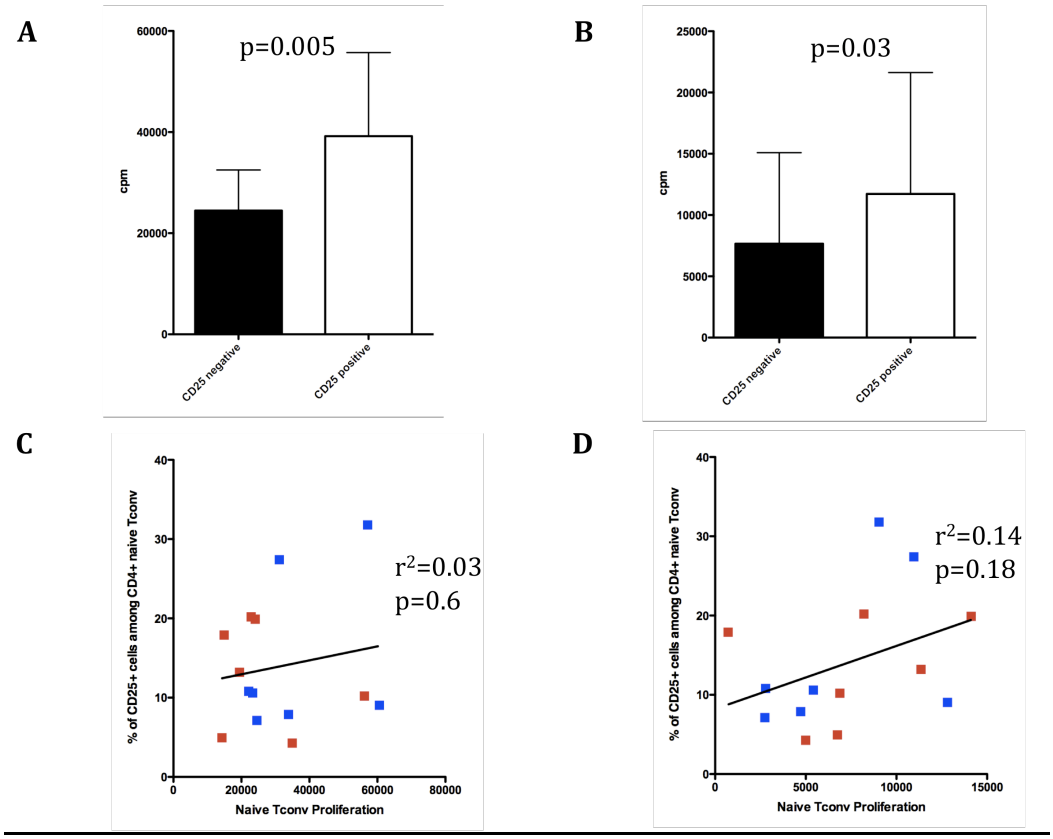

Figure 4.7 Comparison of the proliferation of CD25 negative and positive naïve Tconv populations and the correlation with proliferation

The mean level of proliferation of CD4+ CD25- naïve Tconv versus CD4+ CD25+ naïve Tconv when cells were stimulated with beads @ 1:1 (Figure A) or with beads @ 1:2.5 (Figure B) is shown. Error bars show standard deviation. Data was analysed by a two-tailed Wilcoxon matched-pairs signed rank test. The correlations between the percentage of CD25+ naïve Tconv versus the proliferation of naïve Tconv proliferation when stimulated with beads @ 1:1 (Figure C) and beads @ 1:2.5 (Figure D) were also analysed and assessed by linear regression with p values showing how significantly non-zero the slope was. Blue squares represent protected donors whilst red squares represent susceptible donors. All 16 donors were analysed for all the data in this figure.

between the two donor sets (data not shown).

4.2.8 Apoptosis in total and subpopulations of Tregs

Tregs are prone to apoptosis and require IL-2 to prevent them from this fate (Taams *et al.*, 2001). Ms Garima Garg measured the level of apoptotic cells in the Treg population immediately following the sort (0 hour) or after forty-eight hours in the absence of IL-2 or with 0.2 IU/ml or 2 IU/ml of exogenous IL-2 (Chapter Two, Section 2.8.6). All donors showed low levels of apoptosis at 0 hour, which increased at forty-eight hours, with lower percentages of apoptotic cells correlating with increasing IL-2 concentrations (Figure 4.8A). No significant difference between the donor sets was seen directly after the cells had been isolated (Figure 4.8B). Following forty-eight hours in culture, total Tregs from protected donors showed lower levels of apoptosis compared to susceptible donors, a difference, which reached significance at 2 IU/ml (Figure 4.8). A significant inverse correlation was also seen between the percentage of rTregs and the level of apoptosis, when cells were cultured for forty-eight hours (Figure 4.9). The level of significance decreased with increasing IL-2 concentration, thus demonstrating the ability of IL-2 to prevent apoptosis in these cells.

4.2.9 Suppression of Tconv proliferation in autologous co-cultures

To investigate whether this SNP influenced the sensitivity of naïve Tconv to Treg function, the levels of suppression of Tconv proliferation were

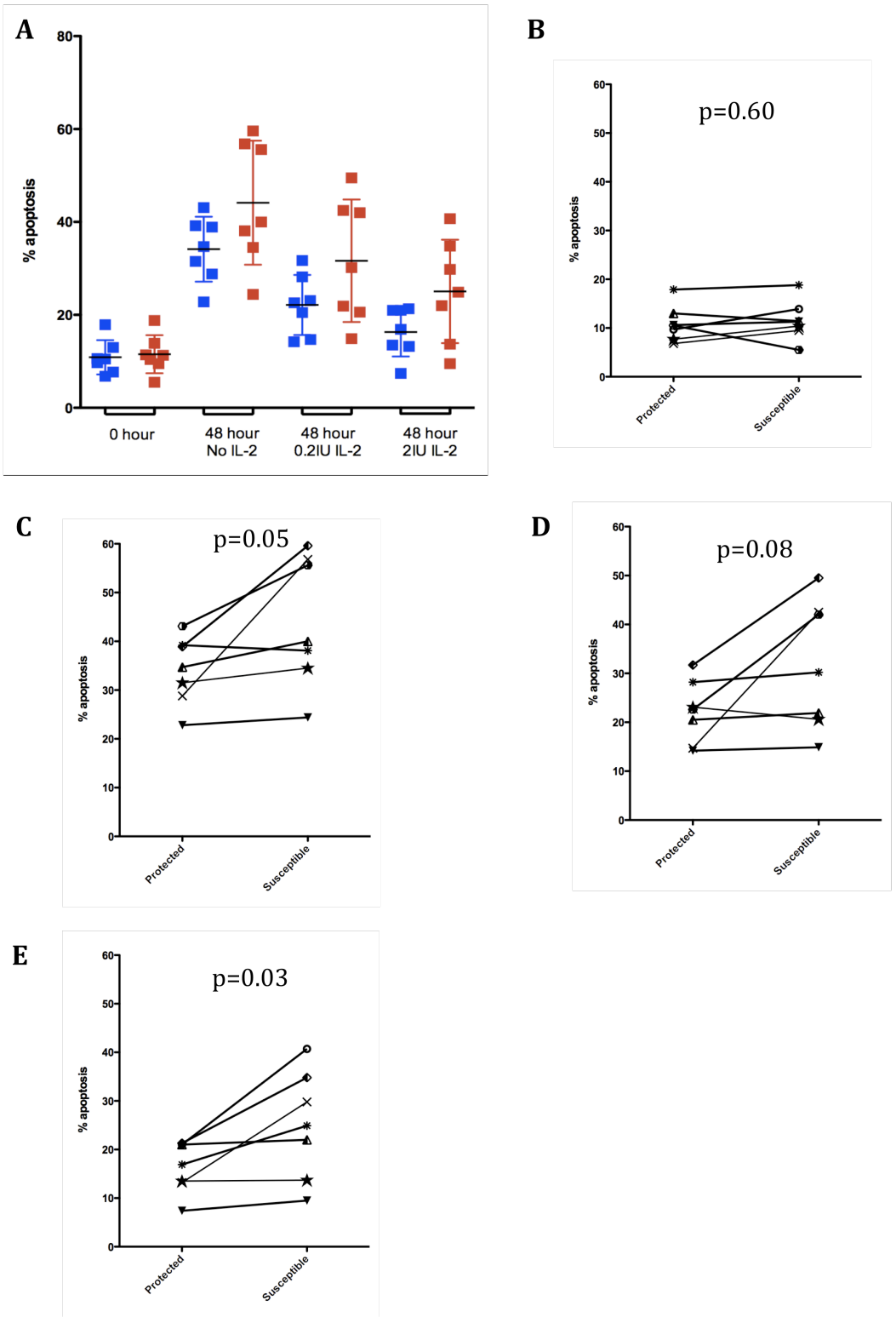

Figure 4.8 Comparison of the percentage of apoptosis in Tregs between sets of donors

The percentage of apoptosis in populations of total Tregs between the sets of donors is shown at each condition (Figure A). Blue squares represent protected donors, whilst red squares represent susceptible donors. The black bars show the mean with error bars showing standard deviation. The percentage of apoptotic cells were analysed directly after isolation of Tregs (Figure B) or after incubation for forty-eight hours in the absence of IL-2 (Figure C) or in the presence of 0.2 IU/ml IL-2 (Figure D) or 2 IU/ml IL-2 (Figure E). Data was analysed by a two-tailed paired t test. Data provided courtesy of Ms Garima Garg.

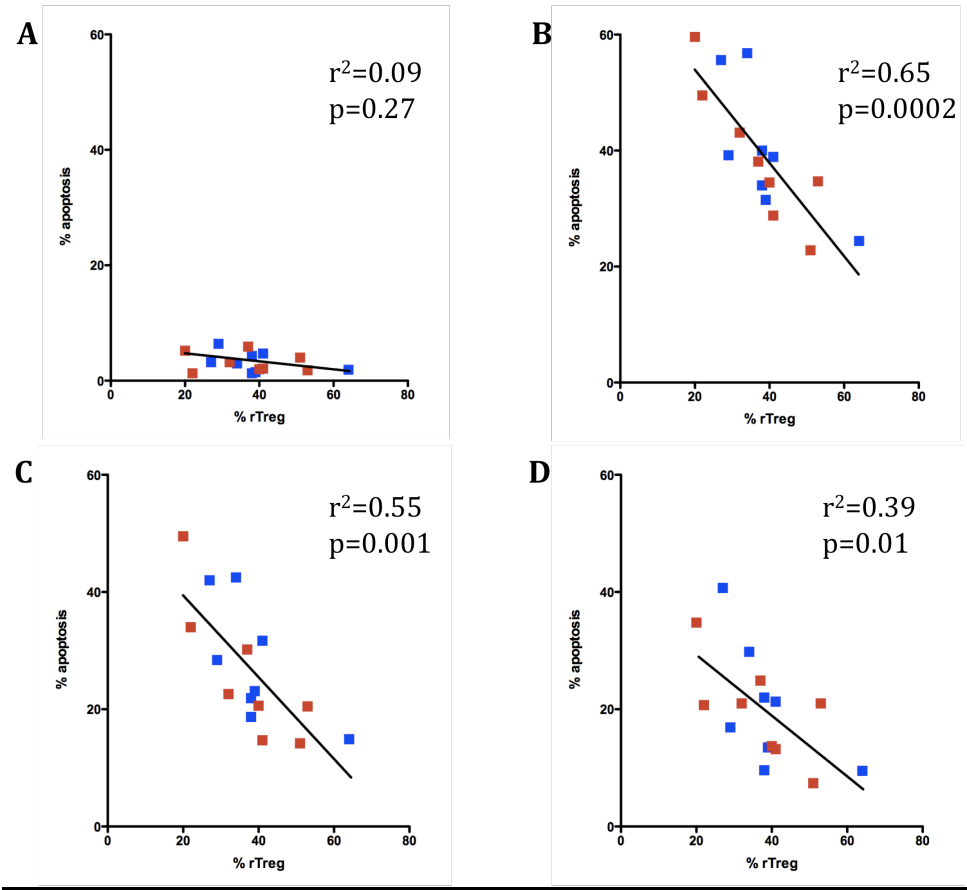

Figure 4.9 Correlation between the level of Treg apoptosis and the percentage of rTregs

The correlation between the percentage of Treg apoptosis and the percentage of rTregs present in the total Treg population for all donors is shown. These were assessed by linear regression with p values showing how significantly non-zero the slope was. Cells were analysed directly after isolation of Tregs (Figure A) or after incubation for forty-eight hours in the absence of IL-2 (Figure B) or in the presence of 0.2 IU/ml IL-2 (Figure C) or 2 IU/ml IL-2 (Figure D). Blue squares represent protected donors whilst red squares represent susceptible donors. Data provided courtesy of Ms Garima Garg.

analysed in two-cell suppression assays using autologous Tregs (Figure 4.10). There were no significant differences in the level of suppression of either memory or naïve Tconv at either signal strength, between donor groups.

4.2.10 Suppression of Tconv proliferation in Standard Treg co-cultures

Two-cell suppression assays comprising of Standard Treg co-cultures with naïve and memory Tconv were also set-up to compare the resistance of these cells to suppression from both donor sets (Figure 4.11). Again, there were no significant differences between the two donor groups at any Tconv:Treg ratio (a Tconv:Treg ratio of 4:1 is shown). Suppression assays were also set-up with Standard Tregs co-cultured with CD25- or CD25+ naïve Tconv (Figure 4.12). No significant differences were seen between the donor groups, although suppression of CD25+ naïve Tconv when stimulated with beads at 1:1, almost reached significance ($p=0.07$). Several data points show negative values of suppression, *i.e.* co-cultures showed higher levels of proliferation than Tconv cultures alone. As discussed in Chapter Three, Section 3.2.2, a sufficient level of cell proliferation is required to be able to calculate suppression accurately. Some of the pairs exhibiting increased proliferation in co-cultures rather than suppression also showed low levels of proliferation of CD25- or CD25+ Tconv alone (Figures 4.5 and 4.6). This problem did not arise in the later genotype-immunophenotype study using this same assay (Chapter Five, Section 5.2.9). Therefore, this may be due to

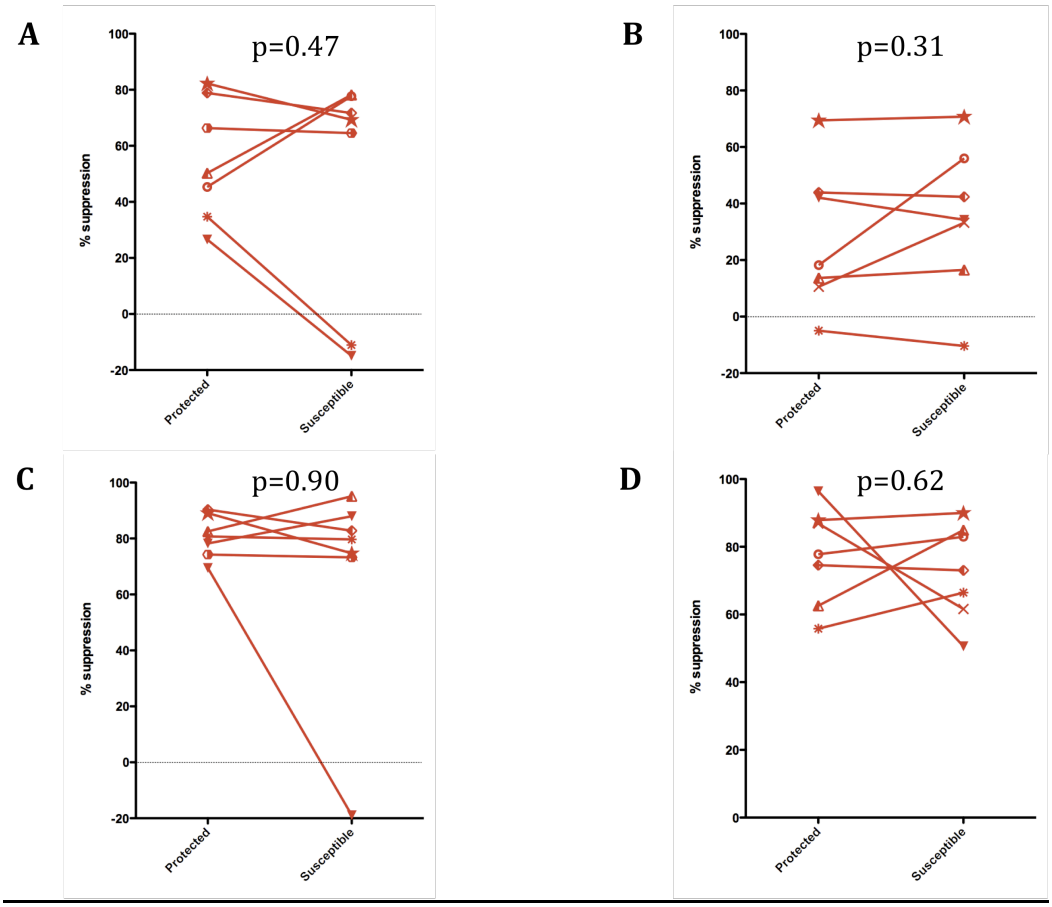

Figure 4.10 Suppression of Tconv proliferation in autologous co-cultures

The mean level of suppression of the proliferation of naïve Tconv when stimulated with beads at 1:1 (Figure A), memory Tconv with beads at 1:1 (Figure B) naïve Tconv stimulated with beads at 1:2.5 (Figure C) and memory Tconv stimulated with beads at 1:2.5 (Figure D) is shown. Data shown are from autologous co-cultures at a Tconv:Treg ratio of 1:1. The dotted line is placed at zero on the y-axis. Suppression was calculated using the formula: % suppression= 100-(counts per minute (cpm) in the presence of Tregs ÷ cpm in the absence of Tregs) x 100). Data in Figures B and D was analysed by a two-tailed paired t test and data in Figures A and C was analysed by a two-tailed Wilcoxon matched-pairs signed rank test.

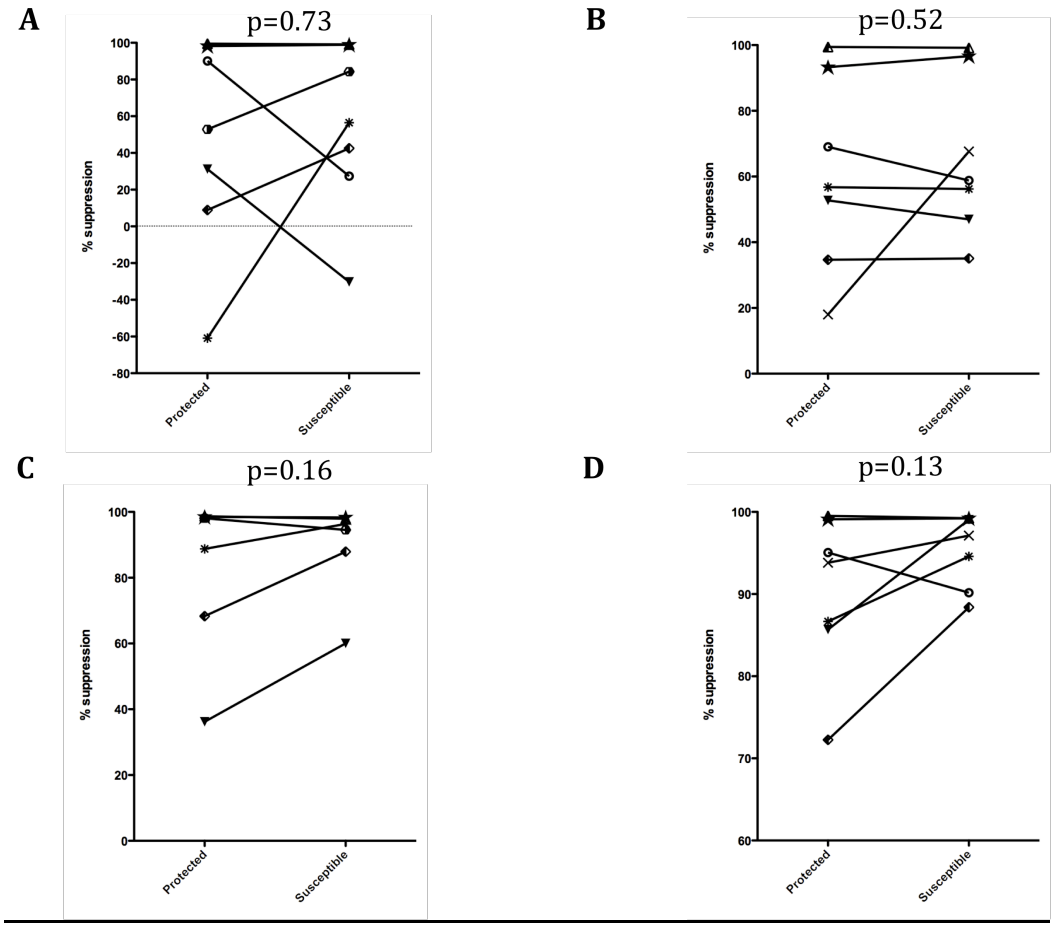

Figure 4.11 ***Suppression of naïve and memory Tconv proliferation in Standard Treg co-cultures***

The mean level of suppression of the proliferation of naïve Tconv when stimulated with beads at 1:1 (Figure A), memory Tconv with beads at 1:1 (Figure B) naïve Tconv stimulated with beads at 1:2.5 (Figure C) and memory Tconv stimulated with beads at 1:2.5 (Figure D) is shown. Data shown are from Standard Treg co-cultures at a Tconv:Treg ratio of 4:1. The dotted line is placed at zero on the y-axis. Suppression was calculated using the formula: % suppression= 100-(counts per minute (cpm) in the presence of Tregs ÷ cpm in the absence of Tregs) x 100). Data was analysed by a two-tailed paired t test.

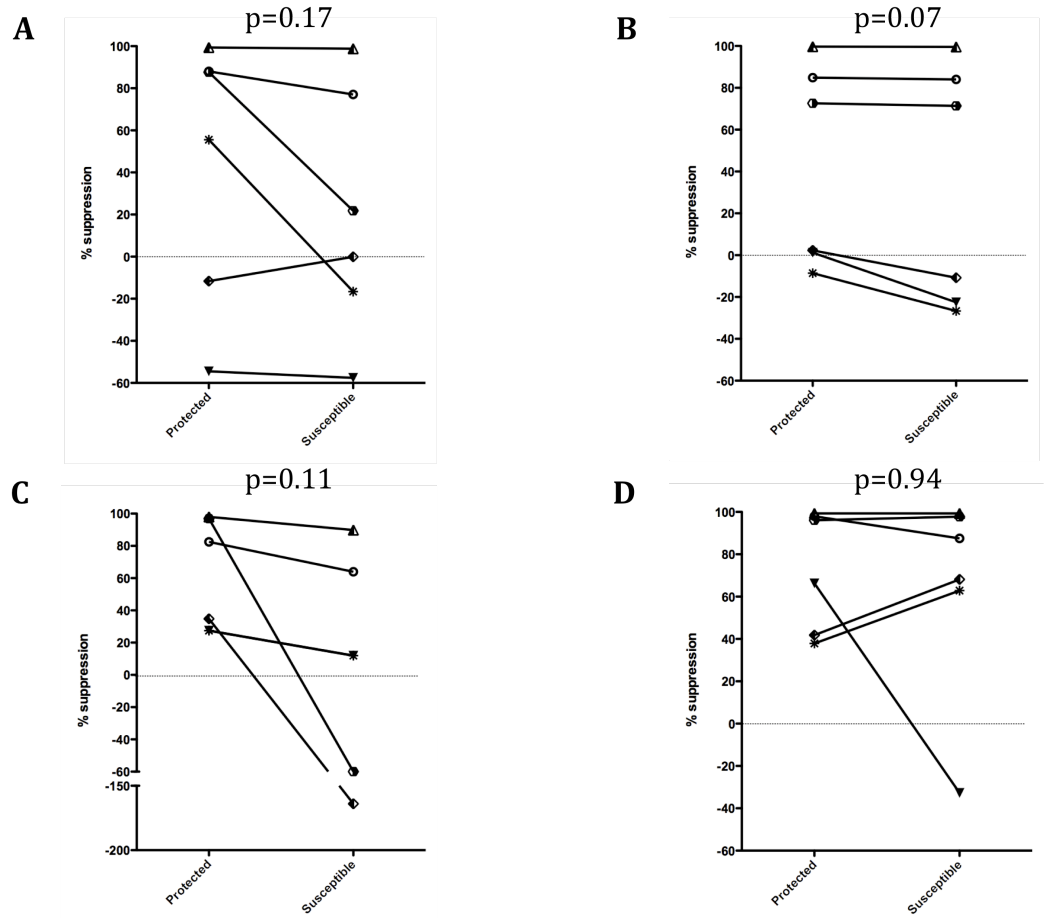

Figure 4.12 ***Suppression of CD25 negative and positive naive Tconv proliferation in Standard Treg co-cultures***

The mean level of suppression of the proliferation of CD25- naïve Tconv stimulated with beads at 1:1 (Figure A), CD25+ naive Tconv stimulated with beads at 1:1 (Figure B) CD25- naïve Tconv stimulated with beads at 1:2.5 (Figure C) and CD25+ naïve Tconv stimulated with beads at 1:2.5 (Figure D) is shown. Data shown are from Standard Treg co-cultures at a Tconv:Treg ratio of 4:1. The dotted line is placed at zero on the y-axis. Suppression was calculated using the formula: % suppression= 100-(counts per minute (cpm) in the presence of Tregs ÷ cpm in the absence of Tregs) x 100). Data in Figures A-

C was analysed by a two-tailed Wilcoxon matched-pairs signed rank test, whilst data in Figure D was analysed by a two-tailed paired t test.

a technical error caused by the unfamiliarity of these assays at this point.

4.2.11 Suppression of CD25- versus CD25+ naïve Tconv proliferation

As there was significantly more proliferation in the CD25+ naïve Tconv subset versus the CD25- naïve Tconv population (Figure 4.7) the level of suppression between these two cell types was compared (Figure 4.13A). No significant difference between the two was seen. Also, the percentage of CD25+ naïve Tconv in the naïve Tconv population was compared to the level of naïve Tconv suppression but no significant correlation was observed (Figure 4.13B).

4.3 Discussion

The purpose of this chapter was to test the hypothesis that SNP.286 influences the ability of CD4+ naïve Tconv to maintain Tregs, thus affecting their suppression.

4.3.1 Implications of the protective allele at SNP.286 P3 in mediating Treg function

The protective allele at SNP.286 is associated with protection from MS (Maier *et al.*, 2009) and JA (Hinks *et al.*, 2009). It has also been reported to be associated with protection from T1D (Maier *et al.*, 2009), although the Protective P3 haplotype is not (Dendrou *et al.*, 2009) (Chapter One, Section 1.13.4 and Section 4.1). How it confers this protection is unclear, but it is

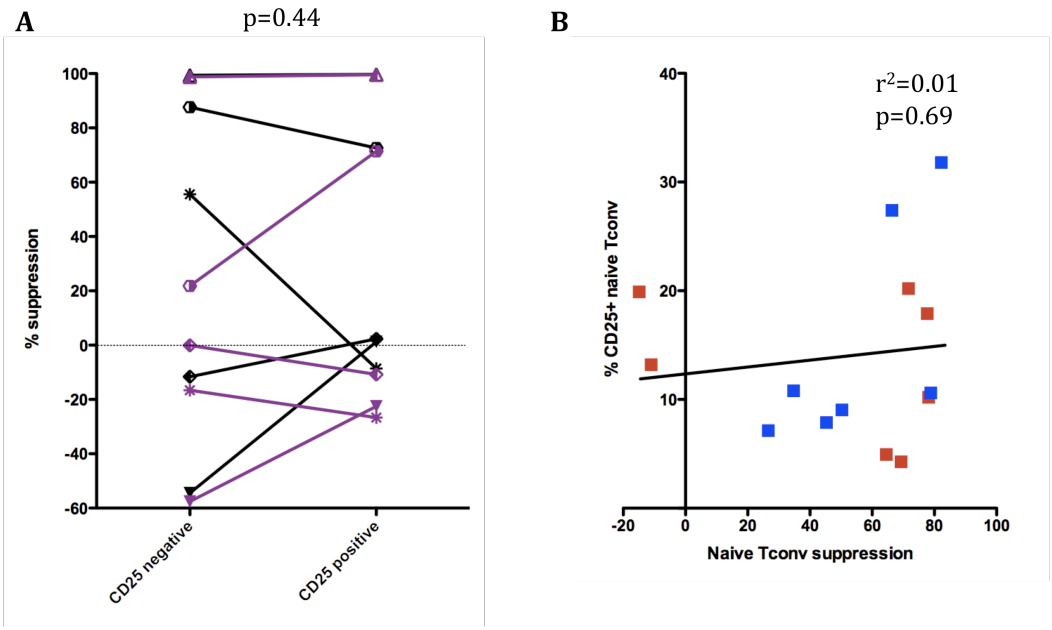

Figure 4.13 Comparison of CD25- and CD25+ naïve Tconv suppression and examination of the relationship between suppression and the percentage of CD25+ naïve Tconv *Figure A shows the comparison of the mean level of suppression of CD25- naïve Tconv compared to CD25+ naïve Tconv proliferation, at a Tconv:Treg ratio of 4:1 when stimulated with beads at 1:1. The black lines showing data from protected donors whilst the purple lines show data from susceptible donors. The dotted line is placed at zero on the y-axis. Data was analysed by a two-tailed paired t test. Figure B shows the correlation between the percentage of CD25+ naïve Tconv and the percentage suppression of naïve Tconv at a Tconv:Treg ratio of 1:1, in cultures stimulated with beads at 1:1 and was assessed by linear regression with p values showing how significantly non-zero the slope was. Blue squares represent protected donors whilst red squares represent susceptible donors. All 16 donors are shown in all data in this figure. Suppression was calculated using the formula: % suppression= 100-(counts per minute (cpm) in the presence of Tregs ÷ cpm*

in the absence of Tregs) x 100).

associated with significantly lower percentages of CD25+ naïve Tconv compared to the susceptible allele (Dendrou *et al.*, 2009), although whether these cells are undergoing activation or whether they are halted in a transitory phase between naïve and memory Tconv in unclear. Neither of these hypotheses could be examined in this chapter, as no difference in the percentage of CD25+ naïve Tconv was evident between the two groups of donors. Therefore, there is as yet no explanation as to why naïve Tconv were found to proliferate more at the strongest stimuli in protected donors than in susceptibles. It would be interesting to observe whether the same result is noted in a larger cohort of donors, or in donors selected for the immunophenotype associated with SNP.286. An increase in naïve Tconv proliferation in these studies may suggest that these cells are less susceptible to a mechanism that prevents them from fully undergoing activation.

Although donors heterozygous for the protective allele at SNP.286 were recruited, the representative immunophenotype (i.e. lower percentages of CD25+ naïve Tconv) was not seen. Indeed, in the study, which first identified this immunophenotype, around two hundred donors were analysed (Dendrou *et al.*, 2009). Therefore in order to conduct the subsequent genotype-immunophenotype study (see Chapter Five) either a vastly higher number of subjects needed to be recruited or cells from the individuals with the genotype of interest required prior analysis, so that those with the representative immunophenotype could be selected. Also,

the immunophenotype associated with the protective allele at SNP.286 is allele-specific dose-dependent (Figure 4.14) (Dendrou *et al.*, 2009). Therefore, where this is seen it would be more beneficial to compare donors homozygous for the allele, rather than heterozygotes. Hence in Chapter Five, the Cambridge BioResource facility was implemented to recruit ten pairs of donors from a cohort of around twelve thousand volunteers, with the genotype and immunophenotype of interest.

Lower levels of Treg apoptosis from protected donors compared to susceptible donors were seen, when these cells were incubated over a forty-eight hour period in the absence or presence of IL-2. An increased susceptibility of Tregs to apoptosis has been identified in T1D patients and in islet auto-antibody-positive at-risk individuals (Glisic *et al.*, 2009, Jailwala *et al.*, 2009, Glisic-Milosavljevic *et al.*, 2007b, Glisic-Milosavljevic *et al.*, 2007a) (Chapter One, Section 1.14.5). Reports suggest that IL-2 deprivation; seen *in vitro* in diabetic patients (Kaye *et al.*, 1986, Zier *et al.*, 1984, Roncarolo *et al.*, 1988) and *in vivo* in the NOD mouse (Yamanouchi *et al.*, 2007, Tang *et al.*, 2008), is largely implicated, leading to the up-regulation of pro-apoptotic genes such as *BIM* (Jailwala *et al.*, 2009). Interestingly, this increase in apoptosis was evident in ROT1D patients but not those with LST1D (Glisic-Milosavljevic *et al.*, 2007b) although IL-2 production in LST1D individuals is also reported to be defective (Kaye *et al.*, 1986, Zier *et al.*, 1984). Studies have also shown the level of suppression seen by Tregs *in vitro* is negatively correlated with the level of apoptosis in these cells (Glisic

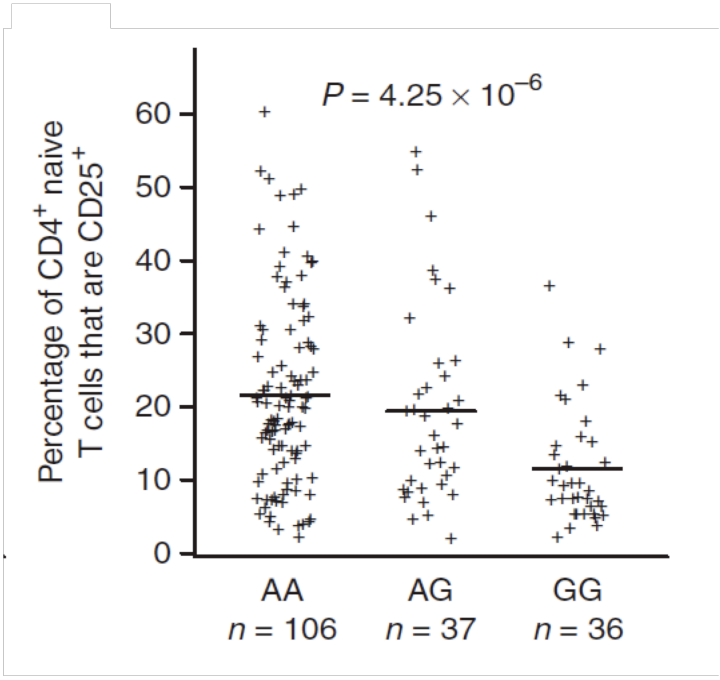

Figure 4.14 The protective allele at SNP.286 is associated with lower percentages of CD25 positive CD4+ naïve Tconv, in an allele-dose-dependent fashion

Data shows the immunophenotype conferred by individuals homozygous for the protective allele at SNP.286 (GG), the susceptible allele at this SNP (AA) or heterozygous (AG) at this SNP. Data was analysed by a one-way ANOVA. Data reproduced from (Dendrou et al., 2009).

et al., 2009). There are, as yet, no reports of the effect of this SNP on Treg phenotype, but it is possible it may be associated with a defect in the IL-2 signalling cascade, although further research would be required to demonstrate this.

Interestingly protected donors had significantly higher percentages of aTregs. Perhaps the increased presence of Tregs, which are already activated, could contribute to autoimmune prevention. Indeed these cells have been shown to be significantly lower in patients with RA, Bechet's Disease (Kim *et al.*, 2012) and SLE (Miyara *et al.*, 2009) compared to control donors (Chapter One, Section 1.8.4). It would be interesting to note whether an increase in aTreg is linked to the protective allele at SNP.286 in a larger cohort of donors, or those with the representative immunophenotype and whether suppression is higher in these individuals. The work in this chapter identified a negative correlation between the percentage of rTregs and apoptosis of total Tregs. Sakaguchi and colleagues showed that upon activation rTregs differentiate into aTregs, whilst the original aTreg population suppress then apoptose (Miyara et al., 2009). Therefore, aTregs may be more susceptible to apoptosis hence why total Treg populations containing more rTregs are less prone to this programme of cell death. Although these cells may be more prone to apoptosis, *in vitro* work by Miyara *et al.* (2009) suggest that they suppress before apoptosing, therefore their function may not be impeded. Also, it may be possible that the immunophenotype associated with the protective allele at SNP.286 may

result in better activation of naïve Tconv and more production of IL-2, thus protecting aTregs from apoptosis.

4.3.2 Conclusions

Although donors heterozygous for the protective allele SNP.286 were selected, the associated immunophenotype was not seen, with both groups of donors showing equal levels of CD25+ naïve Tconv. This compromised the ability to study the influence of the allele at SNP.286 on the ability of Tregs to suppress. This study enforces the importance of either selecting a much larger pool of donors with the particular genotype (as shown in Dendrou *et al.*, 2009) or choosing donors according to these individuals having both the genotype and immunophenotype of interest. This latter option was applied in Chapter Five.

Chapter Five: Examination of the implications of the Type 1 Diabetes-associated Protective P1 *IL-2RA* haplotype, on regulatory T cell maintenance and function

5.1 Introduction

5.1.1 The Protective P1 haplotype

The Protective P1 *IL-2RA* haplotype (discussed in Chapter One, Section 1.13.4, and Chapter Four, Section 4.1) is strongly associated with T1D (Dendrou *et al.*, 2009). As shown in Figure 1.6, this haplotype has the protective alleles at SNP.495 and SNP.286 (but the susceptible allele at SNP.656) (Dendrou *et al.*, 2009). The protective allele at SNP.495 is associated with increased levels of CD25 expression on memory Tconv, in a protective-allele dose-dependent manner (Figure 5.1A) (Dendrou *et al.*, 2009). Also, activated memory Tconv from donors homozygous for the Protective P1 haplotype produced significantly higher levels of IL-2, compared to those from donors homozygous for the Fully Susceptible haplotype (Dendrou *et al.*, 2009). As discussed in Section 4.1, the protective allele at SNP.286, which is also present on this haplotype, is associated with lower percentages of CD25+ naïve Tconv, also in a protective-allele dose-dependent manner (Figure 4.14). Therefore this immunophenotype is associated with both the Protective P1 and Protective P3 haplotypes (Figure 5.1B). An assessment of over 6,500 T1D donors and 8,500 controls

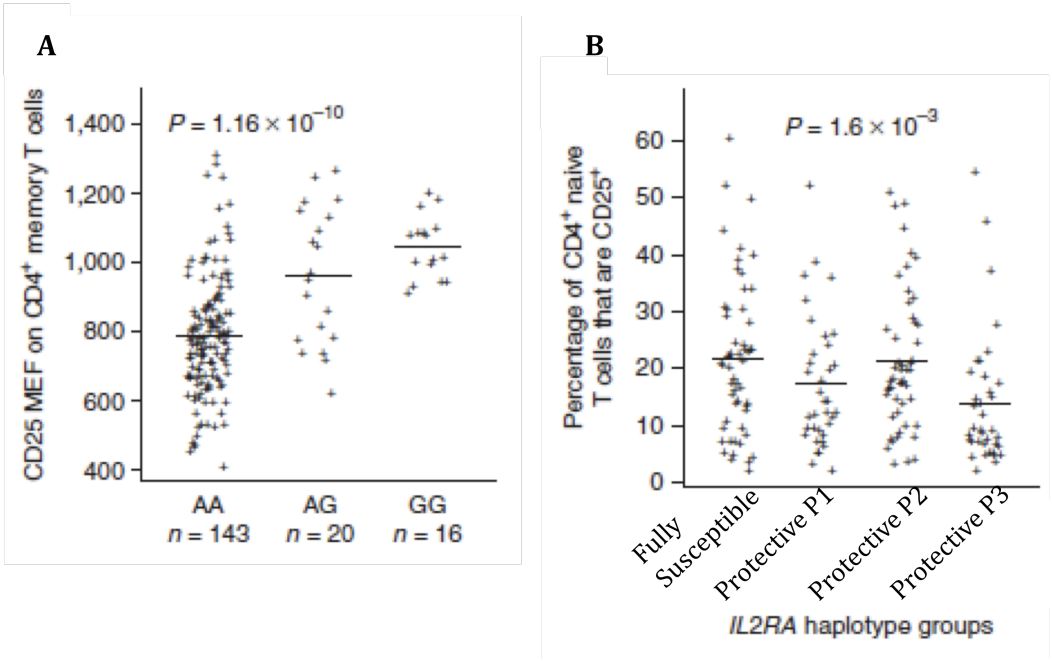

Figure 5.1 The Protective P1 haplotype is associated with higher levels of CD25 expression by CD4+ memory Tconv and lower percentages of CD25+ naïve Tconv

Figure A shows the immunophenotype conferred by individuals homozygous for the protective allele at SNP.495 (GG) or the susceptible allele at this SNP (AA) or heterozygous (AG) at this SNP, with regards to CD4+ memory Tconv CD25 expression. Figure B shows the lower percentage of CD25+ naïve Tconv, associated with the protective allele at SNP.286 is associated with the two haplotypes carrying this protective allele: the Protective P1 haplotype and the Protective P3 haplotype. The data in both Figures A and B were analysed by means of a one-way ANOVA. Data reproduced from (Dendrou et al., 2009).

recruited by the cohort used by Todd and colleagues (the Cambridge BioResource, see Section 5.1.2) found that 7% of diabetic donors were homozygous for the Protective P1 haplotype compared to 11% of controls (Dendrou *et al.*, 2009).

5.1.2 The Cambridge BioResource

To enable research into genes and disease, The Cambridge BioResource was founded to recruit volunteers from the local area (CBR, 2013). This facility was set up in collaboration, among others, with the Cambridge Biomedical Research Centre and the University of Cambridge and by 2012 had recruited over 10,000 donors both with and without health problems. As discussed in Chapter Four, the ability to study the SNP of interest for its influence on the ability of Tregs to suppress was hampered, as the representative immunophenotype was not selected for (see Sections 4.2.3 and 4.3.1). Therefore the Cambridge BioResource was utilised to recruit and pair twenty donors according to not only their genotype but also the immunophenotypes of interest, for work in this chapter. As described in their mission statement, the Cambridge BioResource provides *…'groups of volunteers, tailor-made to the research question in hand'…* (CBR, 2013). This would not have been possible using the local cohort of blood donors at King's College London, as the Protective P1 haplotype is extremely rare (Todd, 2010) and there was a requirement for selecting Protective P1 haplotype homozygous donors, who show the highest levels of memory

Tconv CD25 expression (Figure 5.1A).

5.1.3 Study design

This chapter examines the hypothesis that the Protective P1 haplotype influences the ability of CD4+ Tconv to maintain Tregs, thus affecting their suppression, by means of a genotype-immunophenotype study. A pair-wise analysis was conducted, with each pair consisting of one donor homozygous for the Protective P1 haplotype and the other donor homozygous for the Susceptible haplotype. Although power calculations had shown the minimum number of donors to be recruited was forty (Chapter Four, Section 4.1), for financial reasons, ten pairs of donors were recruited. It should be noted though that our collaborators were able to identify a highly significant difference in the memory Tconv production of IL-2 between those with the Protective P1 haplotype and those with the Fully Susceptible haplotype, from only eight pairs of donors (Dendrou et al., 2009). The cells of interest were isolated from each donor by FACS (as described in Chapter Two, Section 2.8.4 and Chapter Three, Section 3.2.1) and assays were conducted in the same fashion as before (Chapter Four) with proliferation and suppression of each Tconv population measured by a two-cell suppression assay, activated with Dynabeads® at bead:cell ratios of 1:1 and 1:2.5 (Chapter Two, Section 2.8.8 and Chapter Three, Section 3.2.2). In addition to autologous Treg suppression assays, Standard Treg suppression assays were set-up to examine the resistance of Tconv to suppression (see Chapter

Three, Sections 3.2.3 and 3.2.4). Additional work was conducted by Dr. Timothy Tree who examined the phosphorylation of STAT5a (Chapter Two, Section 2.8.3) and Ms Garima Garg who measured FOXP3 maintenance and apoptosis in response to exogenous IL-2 (Chapter Two, Sections 2.8.5 and 2.8.6). This work is presented here, for the purpose of completeness, with their kind permission.

A summary of all procedures conducted on the samples is shown in Figure 5.2, indicating which individual performed each aspect of the work. The first procedures conducted examined cell phenotype and IL-2 signalling, followed by examination of the consequences of IL-2 signalling (FOXP3 maintenance and prevention of apoptosis). Finally, the function of Tregs; in terms of their ability to suppress was investigated. Therefore, each subsequent stage is increasingly complex (Figure 5.3). Statistical analyses were performed as described in Chapter Two, Section 2.8.11 and are given in each figure.

5.2 Results

5.2.1 Donor selection

To avoid any confounding factors of the disease, non-diabetic donors were selected. Each donor homozygous for the Protective P1 haplotype; also possessing CD4+ memory Tconv with the highest levels of CD25 expression, were paired with donors homozygous for the Susceptible haplotype,

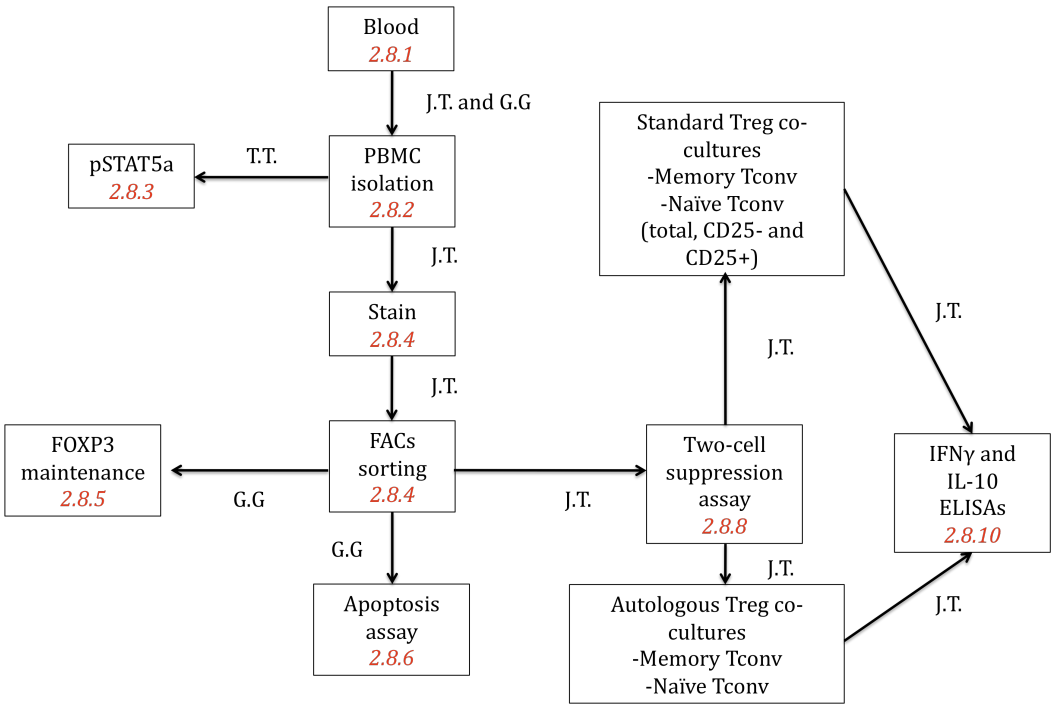

Figure 5.2 Flow chart summarising the procedures conducted on each sample

The above flow chart demonstrates the order and number of all procedures conducted on each pair of samples. The initials of the laboratory worker involved at each stage are given (J.T. Ms Jennifer Tyler, T.T. Dr. Timothy Tree and G.G Ms Garima Garg). The red italics show the section in Chapter Two giving full details of protocols used.

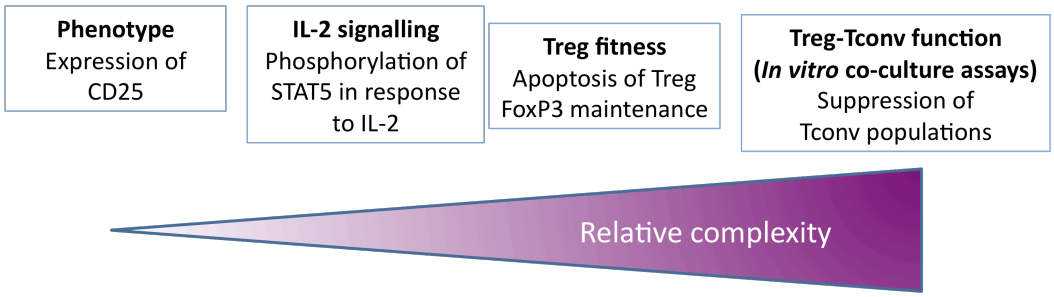

Figure 5.3 The relative complexity of the assays conducted increases with each subsequent procedure

Pair No.	Symbol	Protective Haplotype		Susceptible Haplotype	
		Gender	Age band	Gender	Age band
1	◐	F	35-39	F	30-34
2	◨	M	45-49	M	45-49
3	○	M	40-44	M	40-44
4	■	F	40-44	F	40-44
5	◇	F	45-49	F	45-49
6	●	F	35-39	F	25-29
7	◆	M	30-34	M	30-34
8	△	M	45-49	M	45-49
9	⊗	M	25-29	M	30-34
10	□	F	35-39	F	35-39

Table 5.1 Details of the donors recruited for the study examining the implications of the P1 haplotype on Treg function *M=Male, F=Female*

expressing the lowest levels of CD25 expression (Table 5.1). The donors were gender-matched and paired according to the same or similar age bands. They were also stabilised, as far as possible, for all other known T1D-associated polymorphisms.

5.2.2 Analysis of the frequency and CD25 expression of Tregs and Tconv from data gathered from the STAT5a phosphorylation assay

Following the isolation of PBMC, these cells were either examined in the phosphorylation of STAT5a assay, conducted by Dr. Timothy Tree or were sorted by FACS into the populations used for the Treg suppression assay (see below). The STAT5a phosphorylation assay involved staining the cells with antibodies against CD4, CD25, CD45RA as well as pSTAT5a. This enabled the frequency of the cell populations of interest and their CD25 expression to be examined at this stage. Unfortunately, due to the unsuitability of the anti-CD127 antibody with the fixation buffers used in this assay, Tregs were initially gated on CD4lo CD25hi cells (Figure 5.4A) as described by others (Earle *et al.*, 2005, Lawson *et al.*, 2008) although a more stringent measure, involving the selection of the top 1% of CD25 expressing cells (CD25hi Tregs) (Baecher-Allan *et al.*, 2001) was also used. The percentage of CD4lo CD25hi Tregs from each donor strongly correlated with the percentage of Tregs identified by the CD4+ CD25hi CD127lo phenotype later sorted from the same sample (see Section 5.2.4), demonstrating the likelihood this population are Tregs (Figure 5.4B). Tconv were gated on

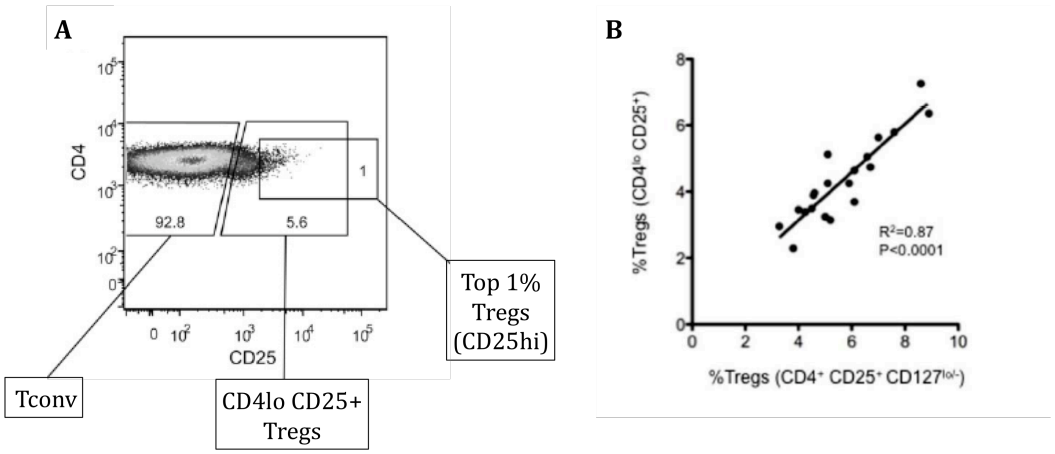

Figure 5.4 Gating strategies of memory and naïve Tconv and Tregs conducted as part of the pSTAT5a assay

Figure A shows the gating of memory and naïve Tconv and Tregs in PBMC samples. As CD127 could not be used as a marker, Tregs were defined as CD4lo CD25+ cells and, more stringently, as the top 1% of CD25 expressing CD4+ cells (CD25hi Tregs). Tconv were classified as CD4+ CD25-/+ cells (Figures A). Memory cells and naïve cells were also divided according to the absence or presence of CD45RA, respectively (data not shown). The percentage of CD4lo CD25+ Tregs isolated from each PBMC sample strongly correlated with the percentage of CD4+ CD25hi CD127lo Tregs later isolated from each sample by FACS (Figure B). The data in Figure B was assessed by linear regression with p values showing how significantly non-zero the slope was. Data provided courtesy of Dr. Timothy Tree.

CD4+ CD25-/+ cells and Memory Tconv selected according to the absence of CD45RA and naïve Tconv selected according to its presence (data not shown).

No differences in the percentage of memory Tconv, naïve Tconv or Tregs were seen between the two donor groups (data not shown). The allele at SNP.495 is associated with the expression of CD25 on memory Tconv (Dendrou *et al.*, 2009). Also, the protective allele at SNP.495 was associated with higher levels of CD25 on CD4+ FOXP3+ cells (Dendrou *et al.*, 2009). However, the necessary application of a Bonferroni Correction showed this difference was non-significant. Therefore, the level of CD25 on memory Tconv and CD4lo CD25+ Tregs in PBMC was measured (Figure 5.5). Protected donors show significantly higher levels of CD25 expression than susceptible donors on both cell types.

5.2.3 Phosphorylation of STAT5a in response to the administration of exogenous IL-2

Dr. Timothy Tree examined the phosphorylation of STAT5a. The concentration of IL-2 administered to the cells was first optimised before these studies were conducted. Tregs showed a higher level of pSTAT5a than Tconv at all IL-2 concentrations tested and pSTAT5a expression appeared to reach a plateau at 10 IU/ml in Tregs (Figure 5.6). Of the Tconv, memory Tconv showed a higher response in terms of pSTAT5a expression than naïve

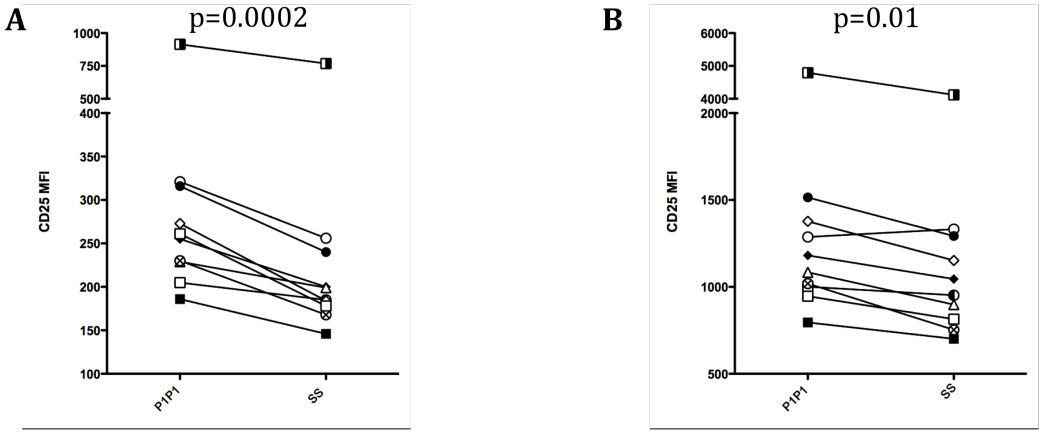

Figure 5.5 CD25 expression on memory Tconv and CD4lo CD25+ Tregs from data gathered during the pSTAT5a assay

The level of expression of CD25 (MFI) on memory Tconv (Figure A) and CD4lo CD25+ Tregs (Figure B) is compared between protected donors (P1P1) and susceptible donors (SS). Data was analysed by a two-tailed paired t test. Data provided courtesy of Dr. Timothy Tree.

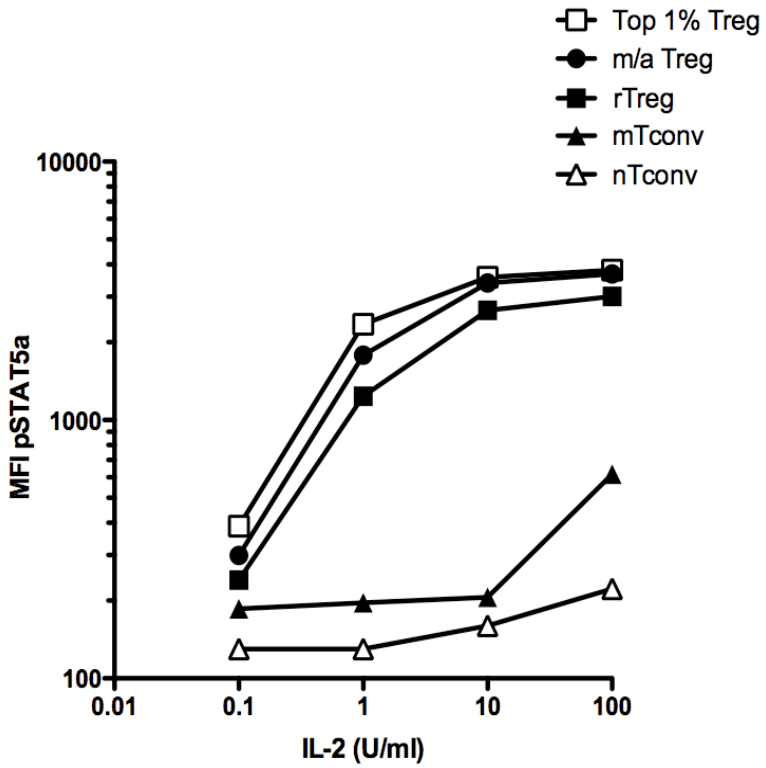

Figure 5.6 Dose-response curve of phosphorylated STAT5a expression in response to increasing IL-2 concentrations

The expression of pSTAT5a (MFI) in response to increasing IL-2 concentrations is shown for one representative individual. The response is shown for the top 1% of Tregs (□), CD45RA- Tregs (includes mTreg and aTregs) (●), CD45RA+ Tregs (aTregs) (■), memory Tconv (▲) and naïve Tconv (△). Data provided courtesy of Dr. Timothy Tree.

Tconv. This order of response in each cell population is probably due to the level of CD25 expression on these cells, as those with characteristically higher levels of this protein show higher responses.

Examination of pSTAT5a expression in memory and naïve Tconv at the lowest levels of IL-2 (0.1 and 1 IU/ml) showed no significant differences between the two donors sets (data not shown). However, at higher IL-2 concentrations (10 and 100 IU/ml), memory Tconv from protected donors showed significantly higher levels of pSTAT5a expression (Figure 5.7) whilst no difference was seen between the donor groups for naïve Tconv. Protected donors also showed significantly higher levels of pSTAT5a expression by CD4lo CD25+ Tregs and CD25hi Tregs, compared to susceptible donors (Figure 5.8). However at higher levels of IL-2 no significant differences in the level of pSTAT5a in Tregs were observed between the donor sets (data not shown).

5.2.4 Isolation of the cells by FACS

Total CD4+ CD25hi CD127lo Tregs, total CD4+ memory Tconv, total CD4+ naïve Tconv and the CD25- and CD25+ subpopulations of CD4+ naïve Tconv were isolated from PBMC from each donor by FACS (Figure 5.9). Memory Tconv were isolated according to the absence of CD45RA, whilst naïve Tconv were isolated according to its presence. After the desired number of naïve Tconv was collected, CD25- and CD25+ naïve Tconv were then isolated.

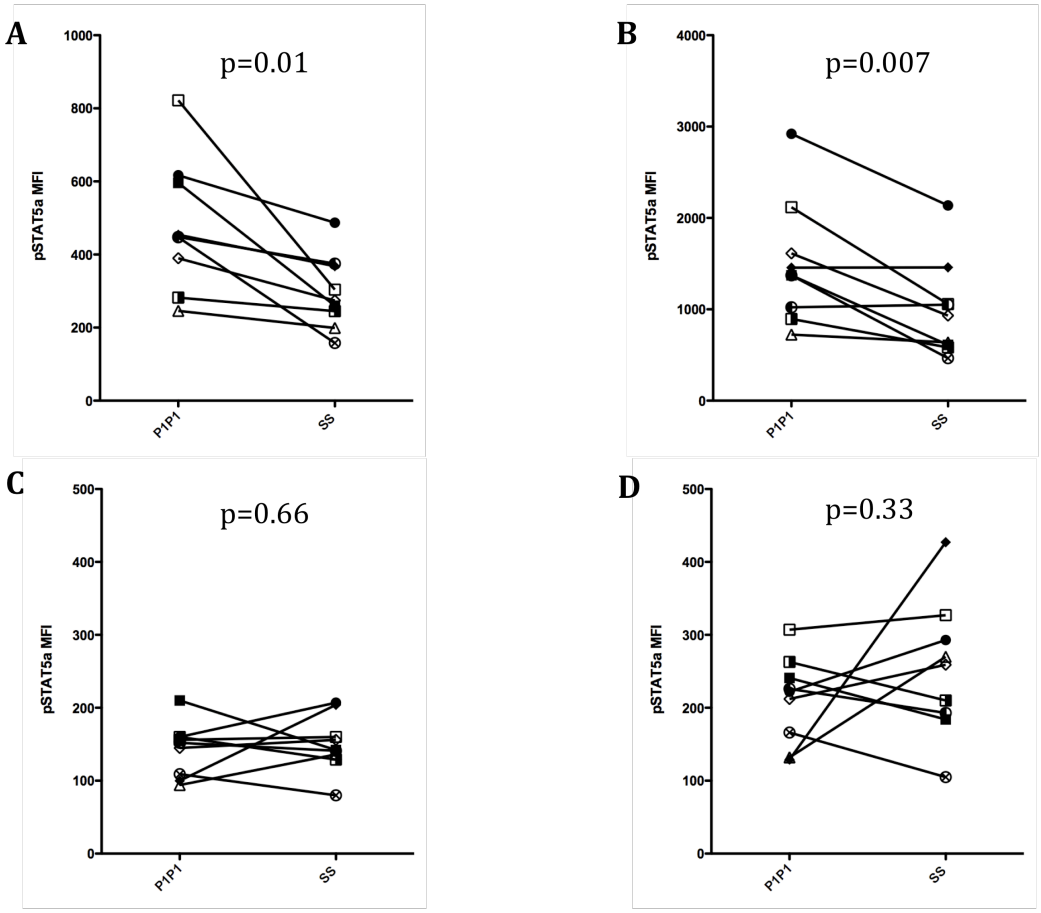

Figure 5.7 pSTAT5a expression in CD4+ Tconv populations exposed to exogenous IL-2

The level of pSTAT5a expression (MFI) in memory Tconv (Figures A and B) and naïve Tconv (Figures C and D) was compared between protected (P1P1) and susceptible (SS) donors. PBMC were incubated with 10 IU/ml (Figures A and C) or 100 IU/ml IL-2 (Figures B and D). Data was analysed by a two-tailed paired t test. Data provided courtesy of Dr. Timothy Tree.

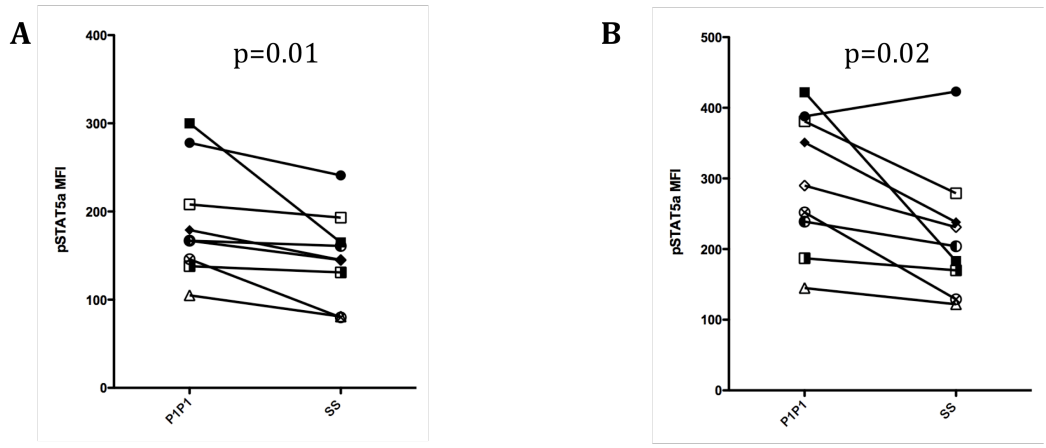

Figure 5.8 pSTAT5a expression in Treg populations exposed to exogenous IL-2

The level of pSTAT5a expression (MFI) was compared in CD4+ CD25lo Tregs (Figure A) and CD25hi Tregs (the top 1% of CD25+ CD4+ cells) (Figure B) between protected (P1P1) and susceptible (SS) donors. PBMC were incubated with 0.1 IU/ml. Data was analysed by a two-tailed paired t test. Data provided courtesy of Dr. Timothy Tree.

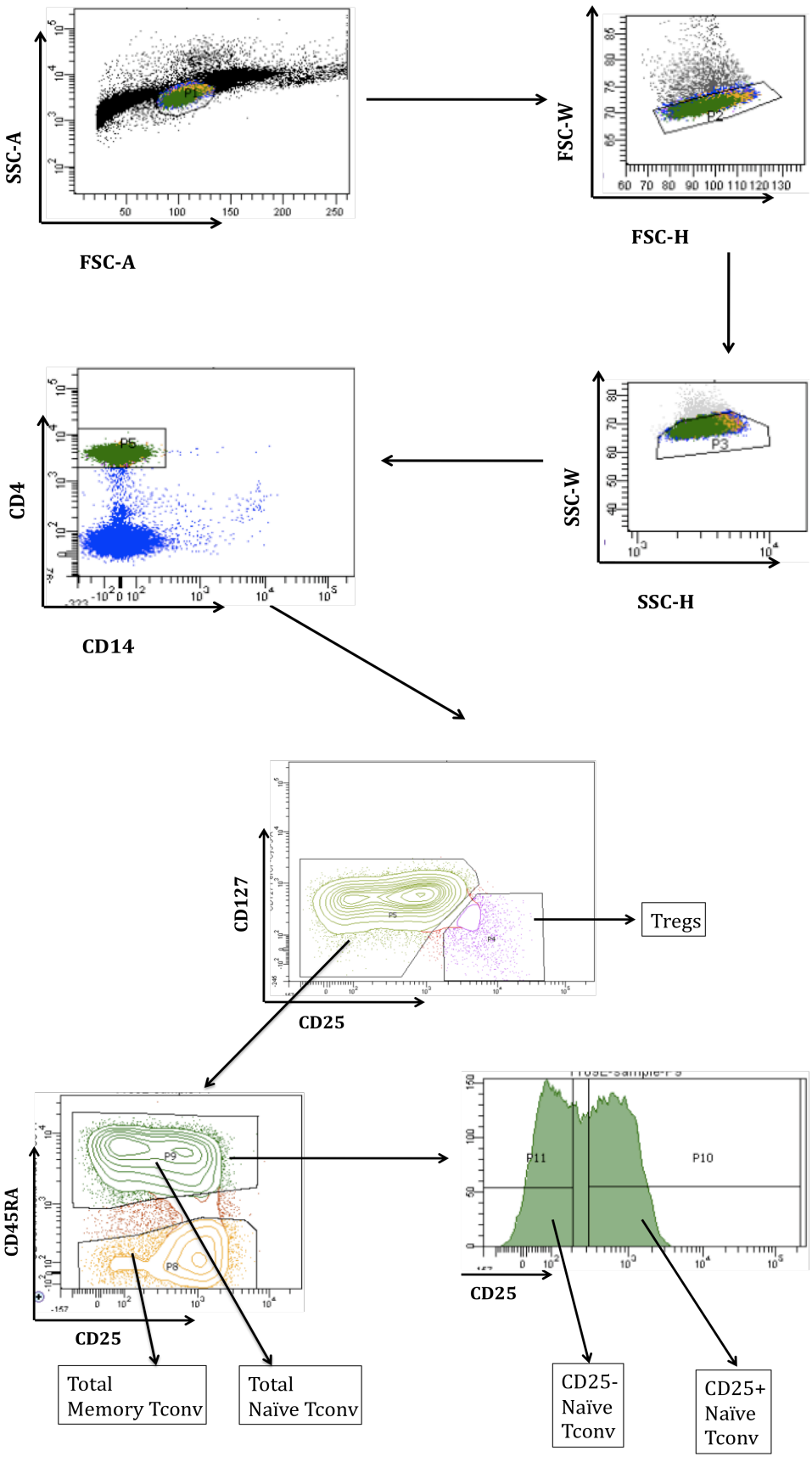
SSC-A
FSC-A
P1
FSC-W
FSC-H
P2
SSC-W
SSC-H
P3
CD4
CD14
P5
CD127
CD25
P5
P4
Tregs
CD45RA
CD25
P9
P8
Total Memory Tconv
Total Naïve Tconv
CD25
P11
P10
CD25- Naïve Tconv
CD25+ Naïve Tconv

Figure 5.9 Gating strategy for the isolation of Tregs, total memory, total naïve and naïve CD25-/+ Tconv

The gating used to isolate cells for the Protective P1 haplotype study is shown. Firstly, lymphocytes were gated and doublets removed. After gating on CD4+ T cells, the Tconv were selected by high expression of CD127 and negative to intermediate expression of CD25. Tregs were isolated according to high expression of CD25 correlating with low levels of CD127. Memory Tconv were isolated according to the absence of CD45RA, whilst naïve Tconv were isolated according to its presence. After the required number of naïve Tconv was collected, the remaining naïve Tconv population was divided according to the absence or presence of CD25.

5.2.5 Analysis of the frequency and CD25 expression of Tregs and Tconv from data gathered from the FACS isolation

As the two protective alleles present at SNP.495 and SNP.286 on the Protective P1 haplotype are associated with higher memory Tconv CD25 expression and lower percentages of CD25+ naïve Tconv, respectively (Dendrou et al., 2009), the frequency of memory and naïve Tconv and these representative phenotypes were examined from the data obtained from the FACS isolation (Figure 5.10). No significant differences between protected and susceptible donors were seen in the frequencies of either the memory or naïve Tconv population. This is in agreement with examinations conducted on cells prepared for the STAT5a phosphorylation assay by Dr. Timothy Tree (Section 5.2.2).

As expected, protected donors showed significantly higher levels of CD25 expression on memory Tconv compared to susceptible donors. However, this difference was not as strongly significant as that identified in memory Tconv from the STAT5a phosphorylation assay. No differences were seen in the percentage of CD25+ naïve Tconv between the two donor groups. This can be explained by the fact our collaborators were only able to select Protective P1 homozygous individuals with the representative immunophenotype associated with SNP.495, but not SNP.286.

The frequency of total Tregs and the three Treg subpopulations (rTreg, mTreg and aTreg; gated as shown in Figure 4.4B) were also investigated

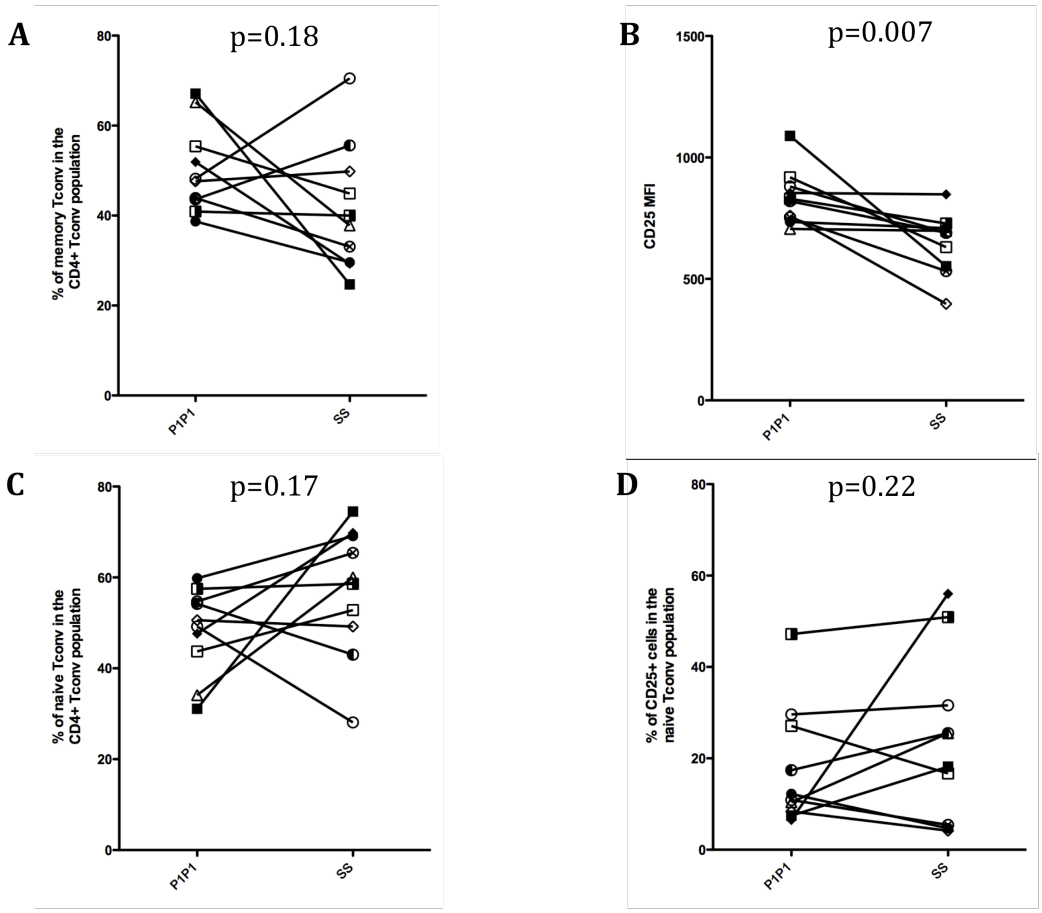

Figure 5.10 Examination of the percentages of memory and naïve Tconv and the immunophenotypes associated with these cell populations, from data gathered during the FACS isolation

The percentage of CD4+ memory Tconv (Figure A), expression of CD25 (MFI) by CD4+ memory Tconv (Figure B), the percentage of CD4+ naïve Tconv (Figure C) and the percentage of CD25+ naïve Tconv (Figure D) were compared between the protective (P1P1) and susceptible (SS) donors. Data from Figures A-C was analysed by a two-tailed paired t test, whilst data from Figure D was analysed by a two-tailed Wilcoxon matched-pairs signed rank test.

(Figure 5.11) but no significant differences were observed between donor groups. The expression of CD25 by each of these cell populations was examined (Figure 5.12), but no significant differences were seen between protected and susceptible donors. These findings are in contrast to the examination of Tregs as part of the STAT5a phosphorylation assay, which found protected donors had significantly higher levels of CD25 expression on Tregs compared to susceptible donors (Figure 5.5). This discrepancy may be due to differences in the procedures (see Section 5.3.2).

5.2.6 Proliferation of the different Tconv populations

The two-cell Treg suppression assays were stimulated with Dynabeads® at bead:cell ratios of 1:1 and 1:2.5. The proliferation of the different Tconv populations was compared to see if there was any difference in the way in which the cells responded to the stimuli (Figures 5.13 and 5.14). However, the levels of proliferation for all Tconv populations were comparable between protected and susceptible donors. The study in Chapter Four (Section 4.2.6) found CD25+ naïve Tconv proliferated at a higher rate compared to CD25- naïve Tconv in Chapter Four. Therefore, the proliferation of these cell populations was also examined in this chapter and found to show the same result (Figure 5.15). The increase in proliferation seen in the CD25+ naïve Tconv population may influence the proliferation of total naïve Tconv. Also the level of memory Tconv CD25 expression might effect the proliferation of these cells. Therefore, the correlations between

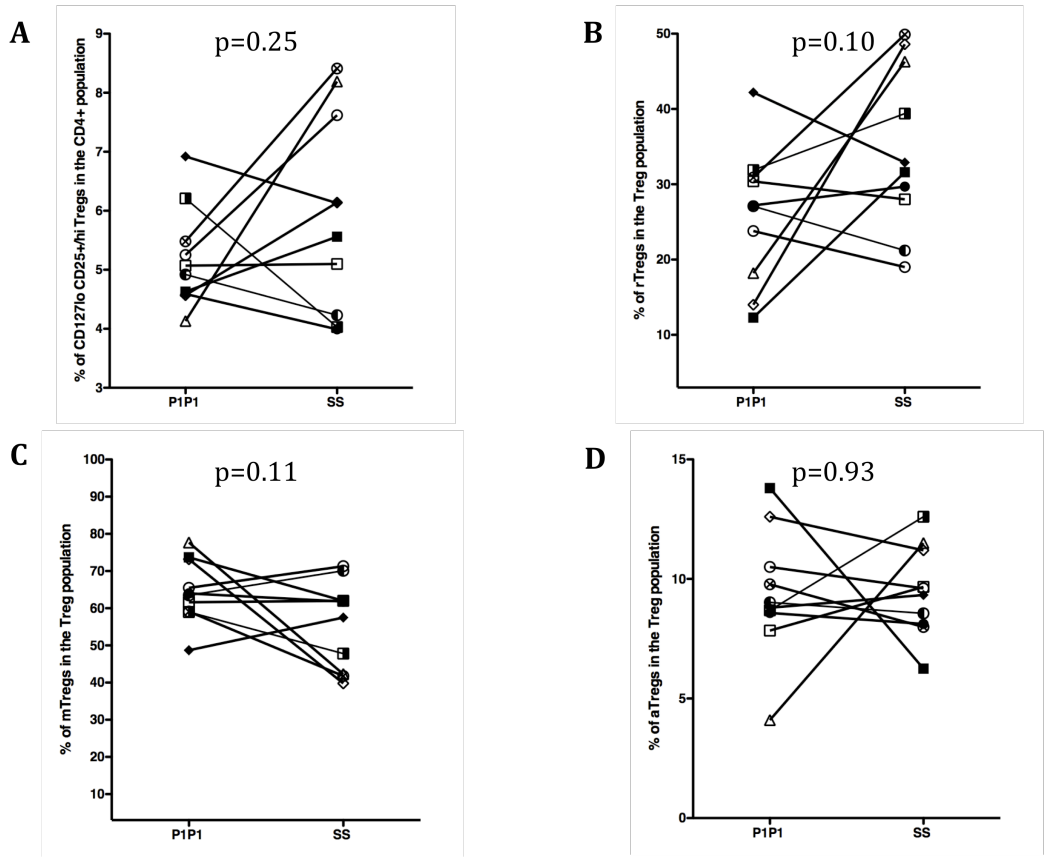

Figure 5.11 Examination of the percentages of total Tregs and the Treg subpopulations from data gathered during the FACS isolation

The percentages of total CD4+ CD25+ CD127lo Tregs (Figure A), rTregs (Figure B), mTregs (Figure C) and aTregs (Figure D) were compared between the protective (P1P1) and susceptible (SS) donors. Data was analysed by a two-tailed paired t test.

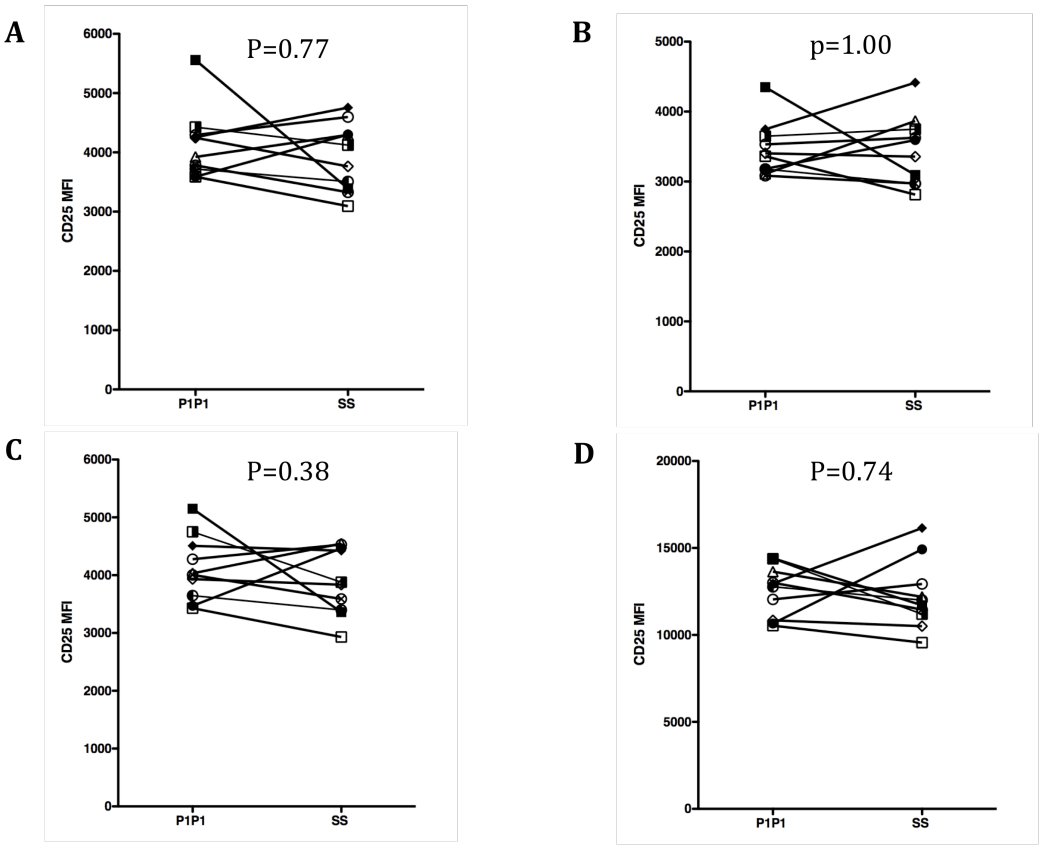

Figure 5.12 Examination CD25 expression on total Tregs and the Treg subpopulations, from data gathered during the FACS isolation

The expression of CD25 (MFI) by total CD4+ CD25+ CD127lo Tregs (Figure A), rTregs (Figure B), mTregs (Figure C) and aTregs (Figure D) were compared between the protective (P1P1) and susceptible (SS) donors. Data from Figures A and B was analysed by a two-tailed Wilcoxon matched-pairs signed rank test, whilst data from Figures C and D was analysed by a two-tailed paired t test.

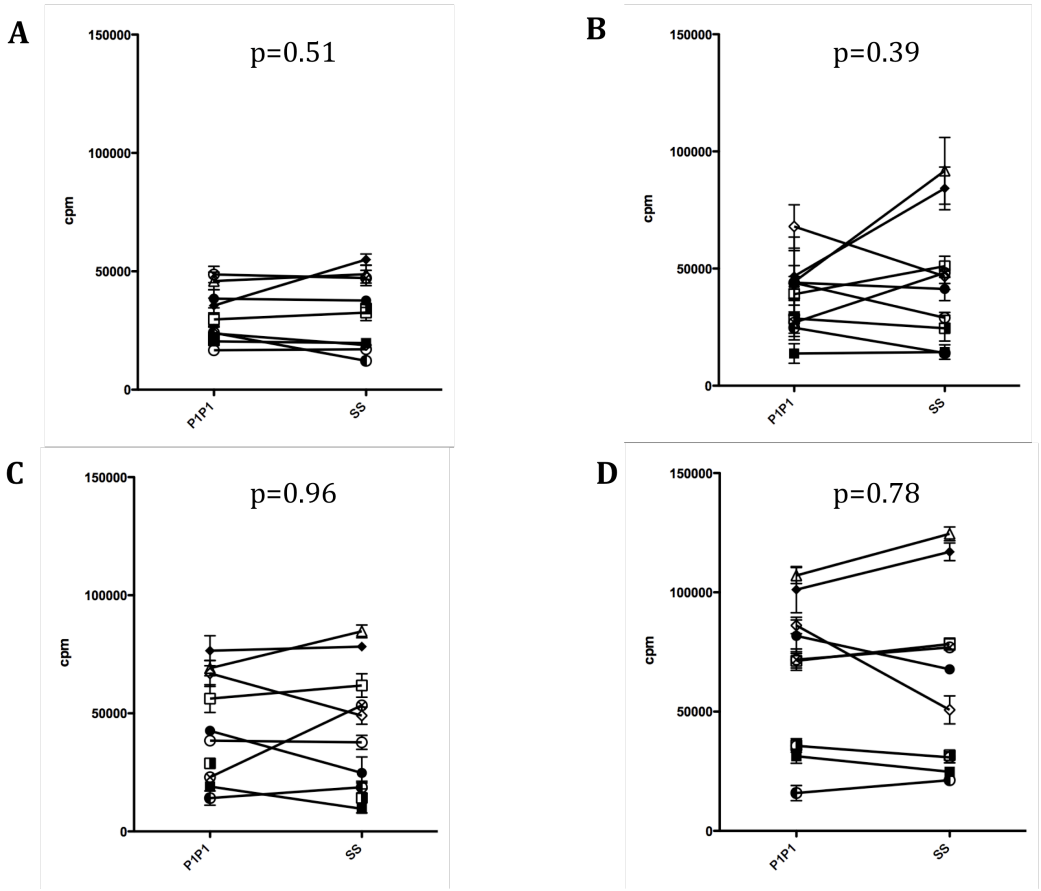

Figure 5.13 Proliferation of CD4+ Tconv subpopulations when stimulated with a bead:cell ratio of 1:1

Proliferation of total memory Tconv (Figure A), total naïve Tconv (Figure B), CD25- naïve Tconv (Figure C) and CD25+ naïve Tconv (Figure D) when stimulated with a bead:cell ratio of 1:1, were compared between the protective (P1P1) and susceptible (SS) donors. Proliferation is shown as mean cpm of 3[H]-thymidine in triplicate wells and error bars show standard deviation. Data was analysed by a two-tailed paired t test.

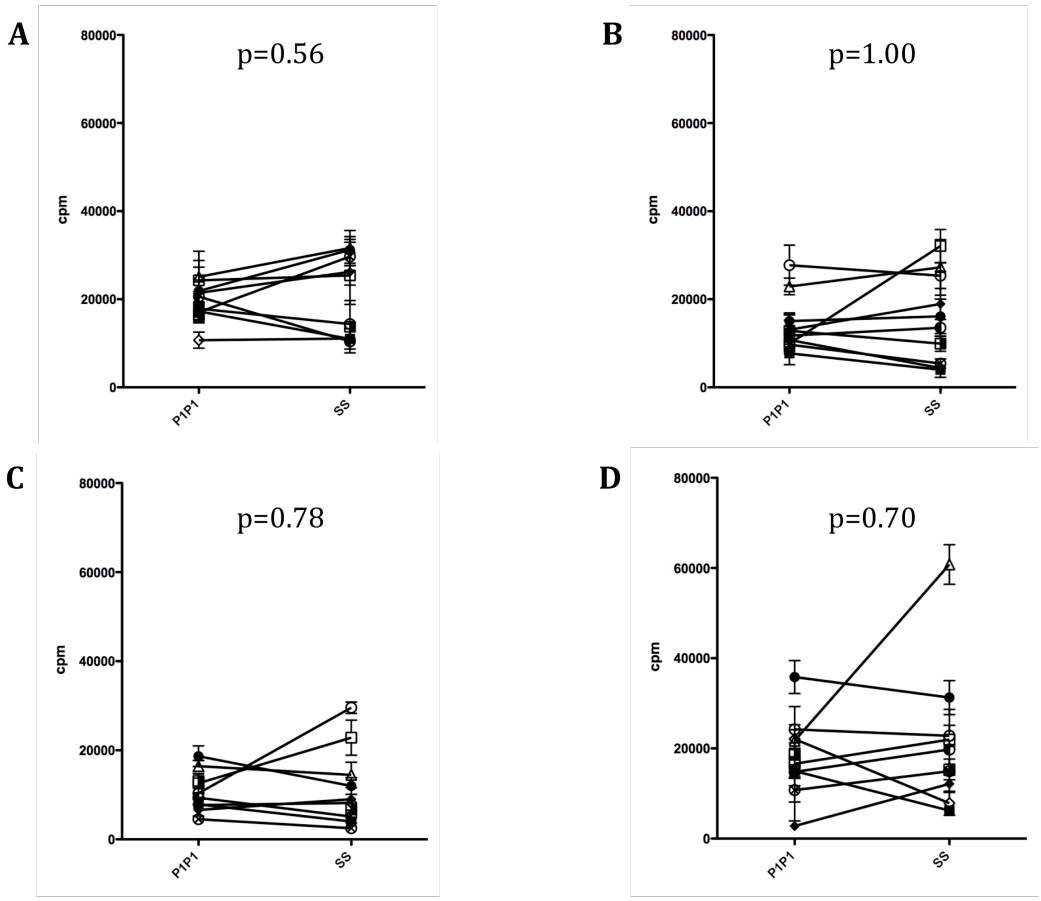

Figure 5.14 Proliferation of CD4+ Tconv subpopulations when stimulated with a bead:cell ratio of 1:2.5

Proliferation of total memory Tconv (Figure A), total naïve Tconv (Figure B), CD25- naïve Tconv (Figure C) and CD25+ naïve Tconv (Figure D) when stimulated with a bead:cell ratio of 1:2.5, were compared between the protective (P1P1) and susceptible (SS) donors. Proliferation is shown as mean cpm of 3[H]-thymidine in triplicate wells and error bars show standard deviation. Data from Figures A, B and D were analysed by a two-tailed Wilcoxon matched-pairs signed rank test, whilst data from Figure C was analysed by a two-tailed paired t test.

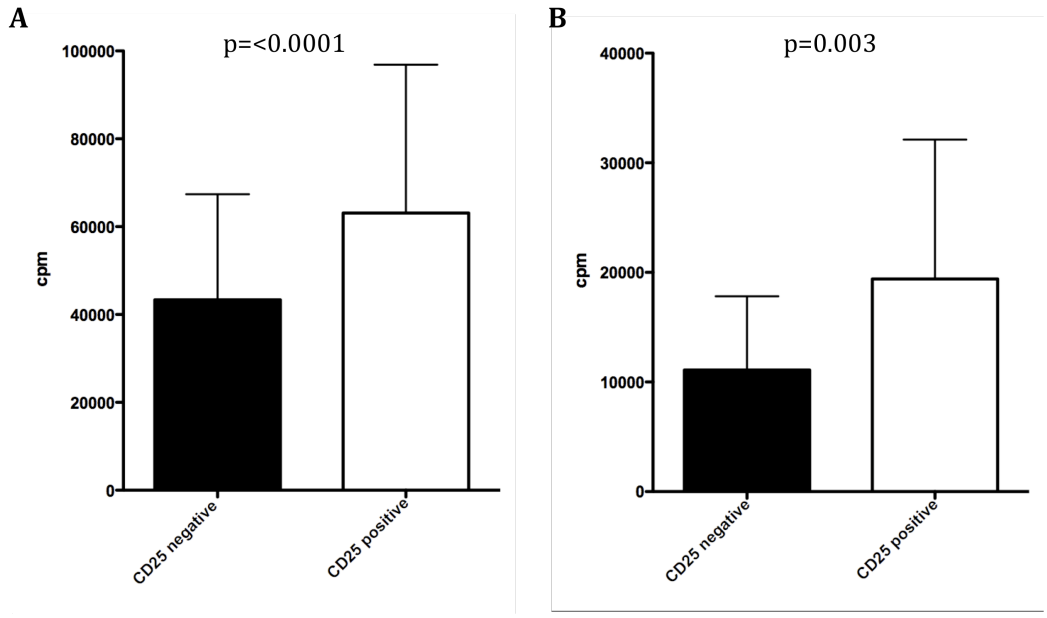

Figure 5.15 Comparison of the proliferation of CD25 negative and positive naïve Tconv populations

The mean level of proliferation of CD4+ CD25- naïve Tconv versus CD4+ CD25+ naïve Tconv when cells were stimulated with beads @ 1:1 (Figure A) or with beads @ 1:2.5 (Figure B) is shown. Figure A shows the response in cells stimulated with a bead:cell ratio of 1:1 and Figure B shows the response at a bead:cell ratio of 1:2.5. Error bars show standard deviation. Data from Figure A was analysed by a two-tailed paired t test, whilst data from Figure B was analysed by a two-tailed Wilcoxon matched-pairs signed rank test. Data was analysed from all 20 donors.

the presence of CD25 and proliferation of these Tconv populations were examined, but no significant relationships were seen (Figure 5.16).

5.2.7 Examination of FOXP3 in thymically-derived Tregs

Tregs were analysed for the expression of FOXP3 and Helios (thought to be a marker of nTregs (Thornton *et al.*, 2010), see Chapter One, Section 1.2.4) by Ms Garima Garg. Tregs were either examined directly after sorting or after incubation for forty-eight hours in the presence of 0, 2 and 20 IU/ml of exogenous IL-2. No differences in the percentage of FOXP3+ or Helios+ Tregs were seen between protected and susceptible donors (data not shown). However, the level of FOXP3 expression in Helios+ Tregs was significantly higher from protected individuals compared to susceptibles, when cells had been incubated for forty-eight hours in the absence of IL-2 or with 2 IU/ml of IL-2 (Figure 5.17). However, no significant difference was seen between protected and susceptible donors at a higher concentration of IL-2 (20 IU/ml).

5.2.8 Apoptosis in Tregs

Ms Garima Garg also examined the percentage of apoptotic Tregs, either directly after sorting or after a forty-eight hour incubation with and without IL-2. However, no significant difference in the percentage of apoptotic Tregs at any condition could be seen between the groups of donors (data not

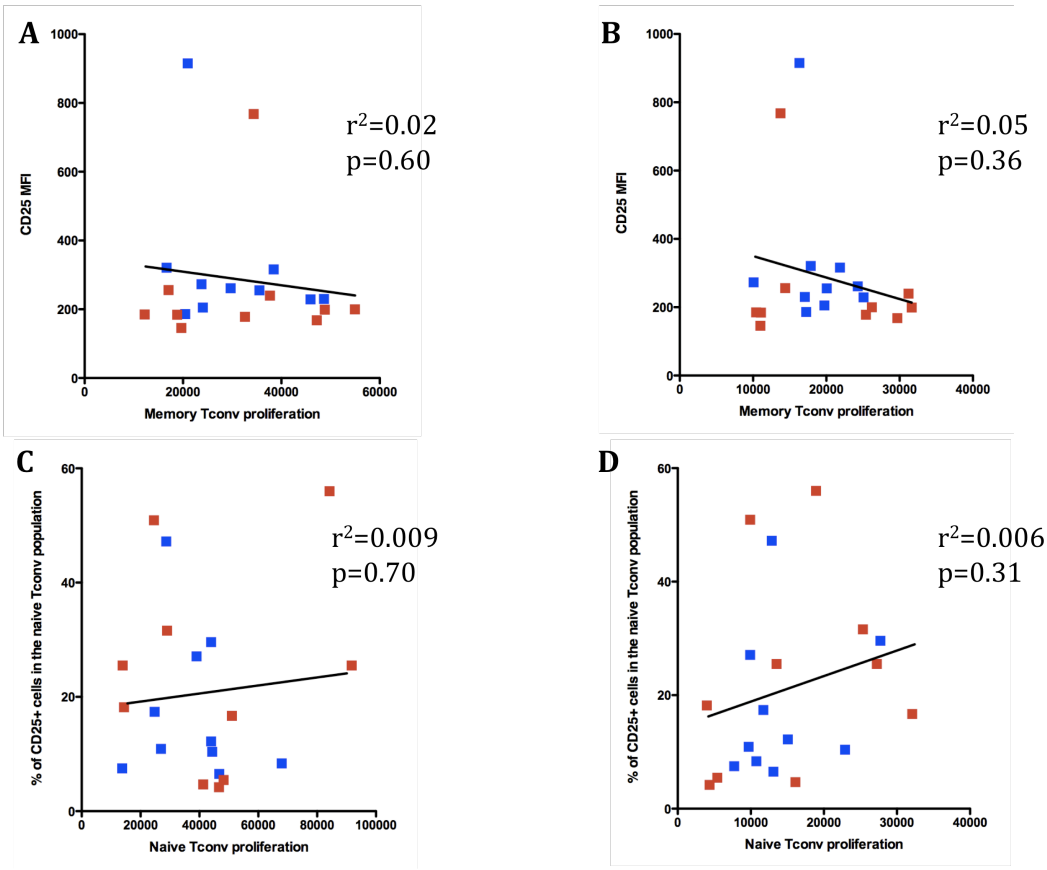

Figure 5.16 Correlation of CD25 with Tconv proliferation

The correlation of memory Tconv CD25 expression and proliferation of these cells when stimulated with beads at 1:1 (figure A) and 1:2.5 (Figure B) was examined. Also the percentage of CD25+ naïve Tconv and proliferation of these cells when stimulated with beads at 1:1 (figure C) and 1:2.5 (Figure D) is shown. Blue squares represent protected donors whilst red squares represent susceptible donors. Correlations were assessed by linear regression with p values showing how significantly non-zero the slope was.

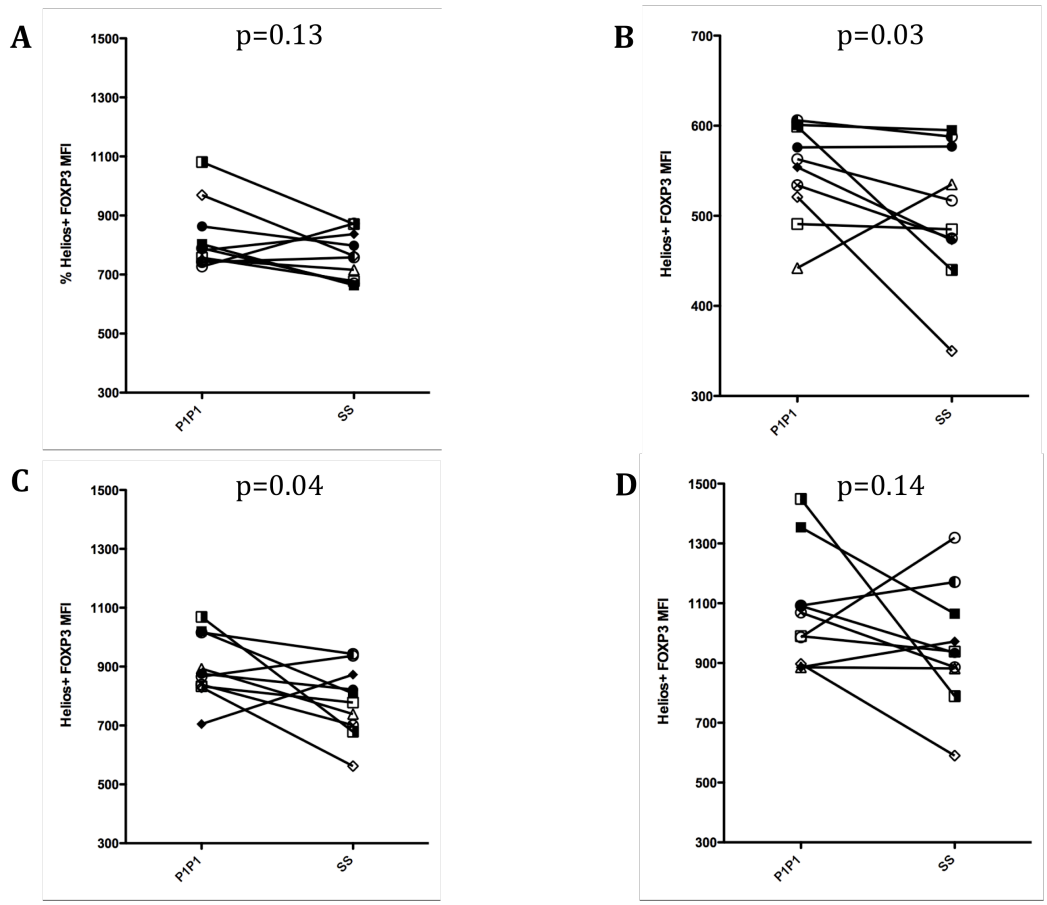

Figure 5.17 FOXP3 expression by Helios+ Tregs

FOXP3 expression (MFI) in Helios+ Tregs is shown immediately after isolation by FACS (Figure A) or following incubation for forty-eight hours in the absence of IL-2 (Figure B) or with 2 IU/ml (Figure C) or 20 IU/ml (Figure D) of IL-2. Protected donors (P1P1) were compared with susceptible (SS) donors. Data was analysed by a one-tailed Wilcoxon matched-pairs signed rank test. Data provided courtesy of Ms Garima Garg

shown).

5.2.9 Suppression of Tconv proliferation in autologous co-cultures

The levels of suppression of Tconv proliferation in co-culture with autologous Tregs were examined (Figure 5.18) using the two-cell suppression assay. Suppression of memory Tconv stimulated with beads at 1:1 was significantly higher in protected donors compared to susceptible donors. At a lower stimulus (beads at 1:2.5) no significant difference was seen between donor sets. The level of suppression of naïve Tconv proliferation at both stimuli was comparable between the groups of donors.

5.2.10 Suppression of Tconv proliferation in Standard Treg co-cultures

Treg suppression assays utilising Standard Tregs were set up for all four Tconv populations (memory, naïve and CD25-/+ naïve Tconv). No significant differences in suppression were seen between the two donor groups for any Tconv population at any Tconv:Treg ratio at either stimulus (a Tconv:Treg ratio of 4:1 is shown) (Figures 5.19 and 5.20).

5.2.11 Suppression of CD25- versus CD25+ naïve Tconv proliferation

CD25+ naïve Tconv show significantly higher levels of proliferation than CD25- naïve Tconv population (Figure 4.7 and Figure 5.15). The level of suppression between these two cell types was compared, however in

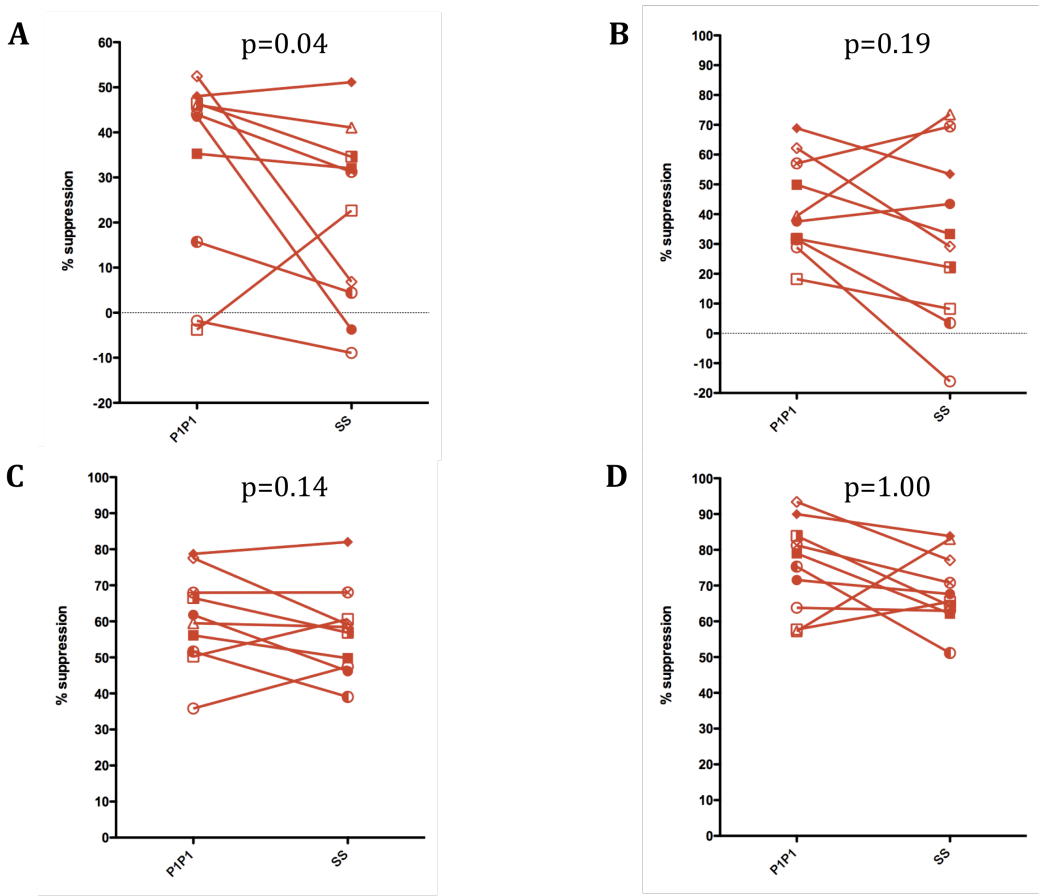

Figure 5.18 Suppression of Tconv proliferation in autologous co-cultures

The mean level of suppression of memory Tconv proliferation when stimulated with beads at 1:1 (Figure A), naive Tconv stimulated with beads at 1:1 (Figure B), memory Tconv with beads at 1:2.5 (Figure C) and naive Tconv stimulated with beads at 1:2.5 (Figure D) is shown. Protected donors (P1P1) were compared with susceptible (SS) donors. Data shown are from autologous co-cultures at a Tconv:Treg ratio of 1:1. The dotted line is placed at zero on the y-axis. Suppression was calculated using the formula: % suppression= 100-(counts per minute (cpm) in the presence of Tregs ÷ cpm in the absence of Tregs) x 100). Data from Figure A was analysed using one-tailed Wilcoxon matched-pairs signed rank test, whilst data from Figures B-D was analysed using one-tailed paired t test.

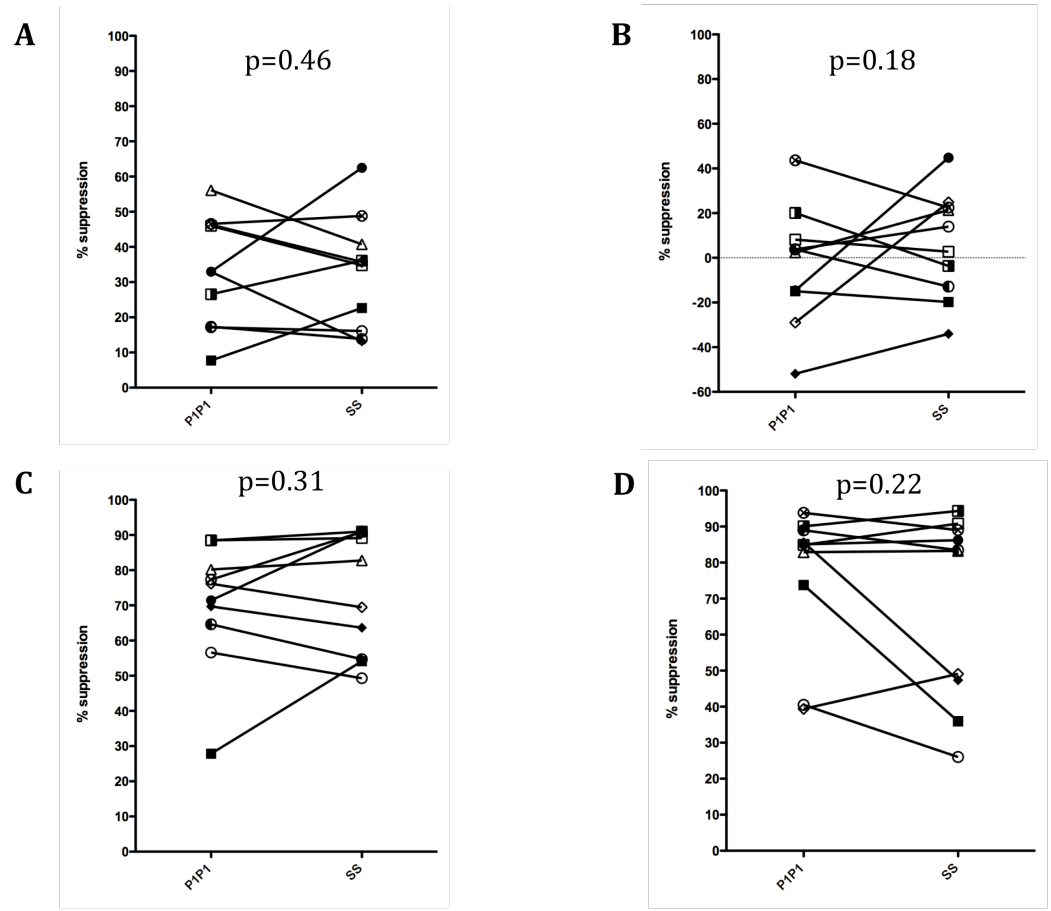

Figure 5.19 Suppression of memory and naïve Tconv proliferation in Standard Treg co-cultures

The mean level of suppression of memory Tconv proliferation when stimulated with beads at 1:1 (Figure A), naive Tconv stimulated with beads at 1:1 (Figure B), memory Tconv with beads at 1:2.5 (Figure C) and naive Tconv stimulated with beads at 1:2.5 (Figure D) is shown. Protected donors (P1P1) were compared with susceptible (SS) donors. Data shown are from Standard Treg co-cultures at a Tconv:Treg ratio of 4:1. The dotted line is placed at zero on the y-axis. Suppression was calculated using the formula: % suppression= 100-(counts per minute (cpm) in the presence of Tregs ÷ cpm in the absence of Tregs) x 100). Data from Figures A and B was analysed using one-tailed

paired t test, whilst data from Figures C and D was analysed using one-tailed Wilcoxon matched-pairs signed rank test.

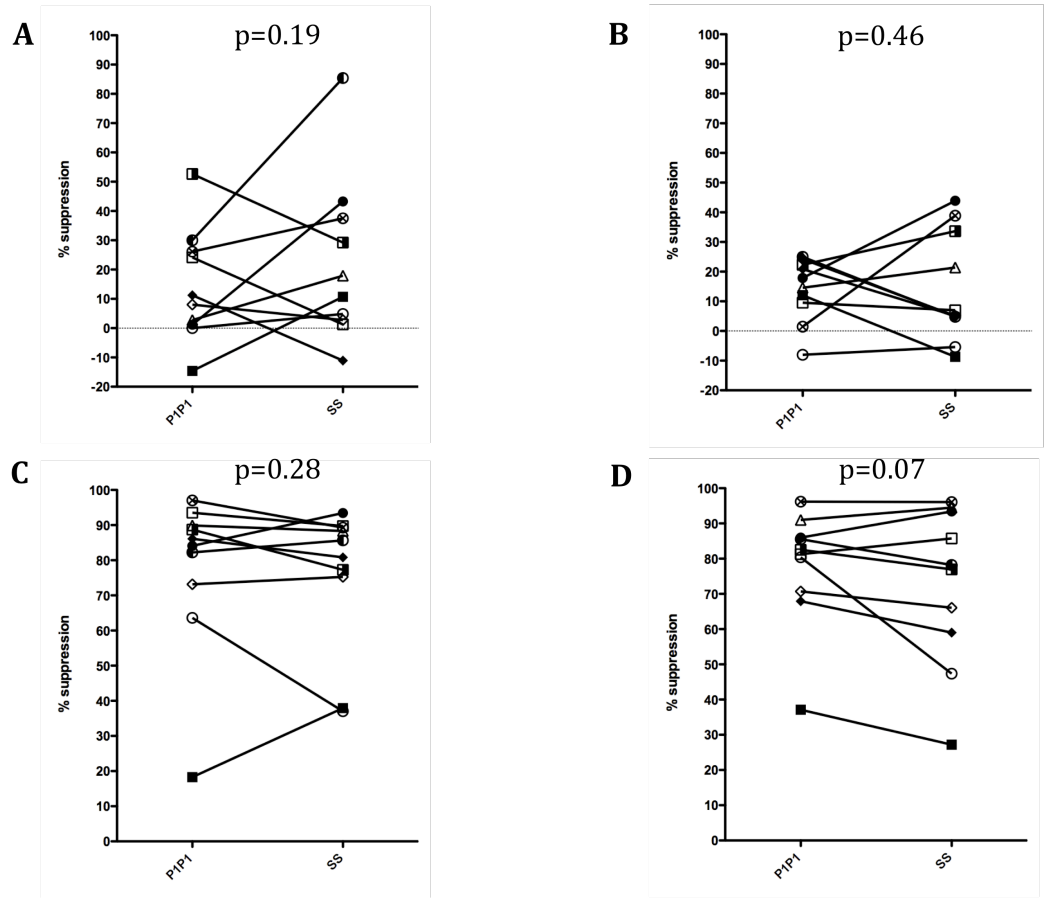

Figure 5.20 ***Suppression of CD25 negative and positive naive Tconv proliferation in Standard Treg co-cultures***

The mean level of suppression of CD25- naïve Tconv proliferation when stimulated with beads at 1:1 (Figure A), CD25+ naïve Tconv stimulated with beads at 1:1 (Figure B), CD25- naive Tconv with beads at 1:2.5 (Figure C) and CD25+ naive Tconv stimulated with beads at 1:2.5 (Figure D) is shown. Protected donors (P1P1) were compared with susceptible (SS) donors. Data shown are from Standard Treg co-cultures at a Tconv:Treg ratio of 4:1. The dotted line is placed at zero on the y-axis. Suppression was calculated using the formula: % suppression= 100-(counts per minute (cpm) in the presence of Tregs ÷ cpm in the absence of Tregs) x 100). Data from Figures A and B was analysed using one-tailed paired t test, whilst data from Figures C and D was

analysed using one-tailed Wilcoxon matched-pairs signed rank test.

concordance with results in Chapter Four (Section 4.2.11), no significant differences were observed (Figure 5.21). Also, the relationship between the levels of CD25 expression on memory Tconv or the percentage of CD25+ naïve Tconv and autologous suppression of these cells was examined (Figure 5.22) but no significant correlations were found.

5.2.12 Cytokine production by memory Tconv cultured alone or with Tregs

Protected individuals showed significantly higher levels of memory Tconv suppression in autologous co-cultures stimulated with a bead:cell ratio of 1:1. Therefore the supernatants from these cultures (taken prior to the addition of 3[H]-thymidine) were assessed for production of IFNγ and IL-10 (Chapter Two, Section 2.8.10). Supernatants from Standard Treg co-cultures with memory Tconv were also examined as were supernatants from naïve autologous and Standard Treg co-cultures, all stimulated with a bead:cell ratio of 1:1, as a comparison as these had shown no significant difference between donor groups. No significant difference in the level of memory Tconv IFNγ production when cultured alone was seen between the sets of donors (Figure 5.23). Surprisingly, memory Tconv from protected donors produced significantly higher levels of this cytokine when in co-culture with autologous Tregs, although this was not seen in Standard Treg co-cultures. Also, there were no significant differences between protected and susceptible donors in the level of IFNγ suppression in either autologous

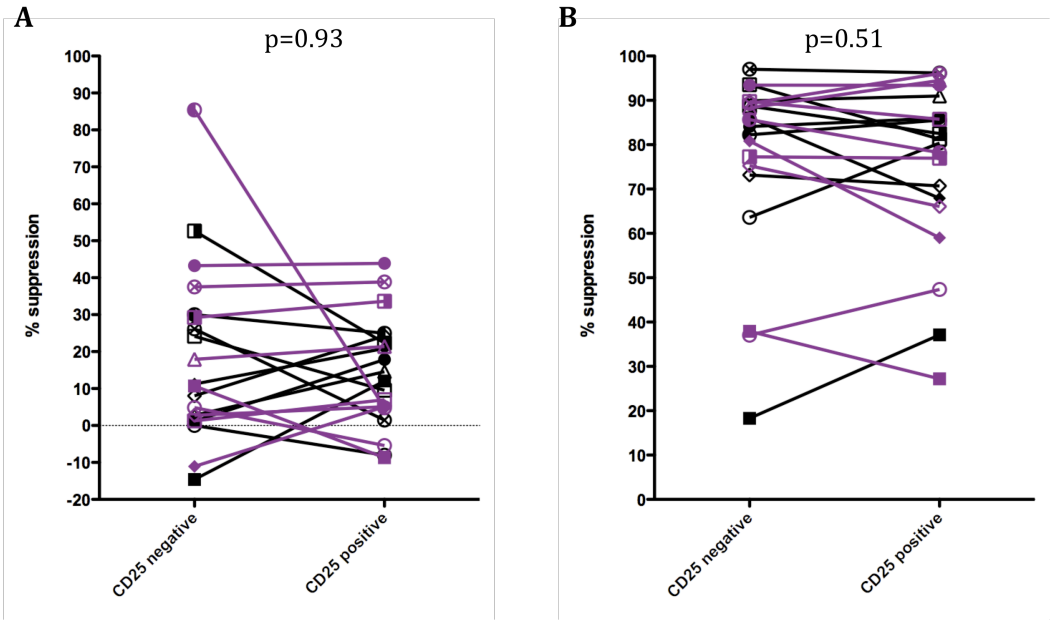

Figure 5.21 Comparison of CD25- and CD25+ naïve Tconv suppression

The comparison of the mean level of suppression of CD25- naïve Tconv compared to CD25+ naïve Tconv proliferation, at a Tconv:Treg ratio of 4:1 when stimulated with beads at 1:1 (Figure A) and at 1:2.5 (Figure B) was examined. All 20 donors are shown with black lines representing data from protected donors whilst the purple lines show data from susceptible donors. The dotted line is placed at zero on the y-axis. Suppression was calculated using the formula: % suppression= 100-(counts per minute (cpm) in the presence of Tregs ÷ cpm in the absence of Tregs) x 100). Data was analysed by a two-tailed Wilcoxon matched-pairs signed rank test.

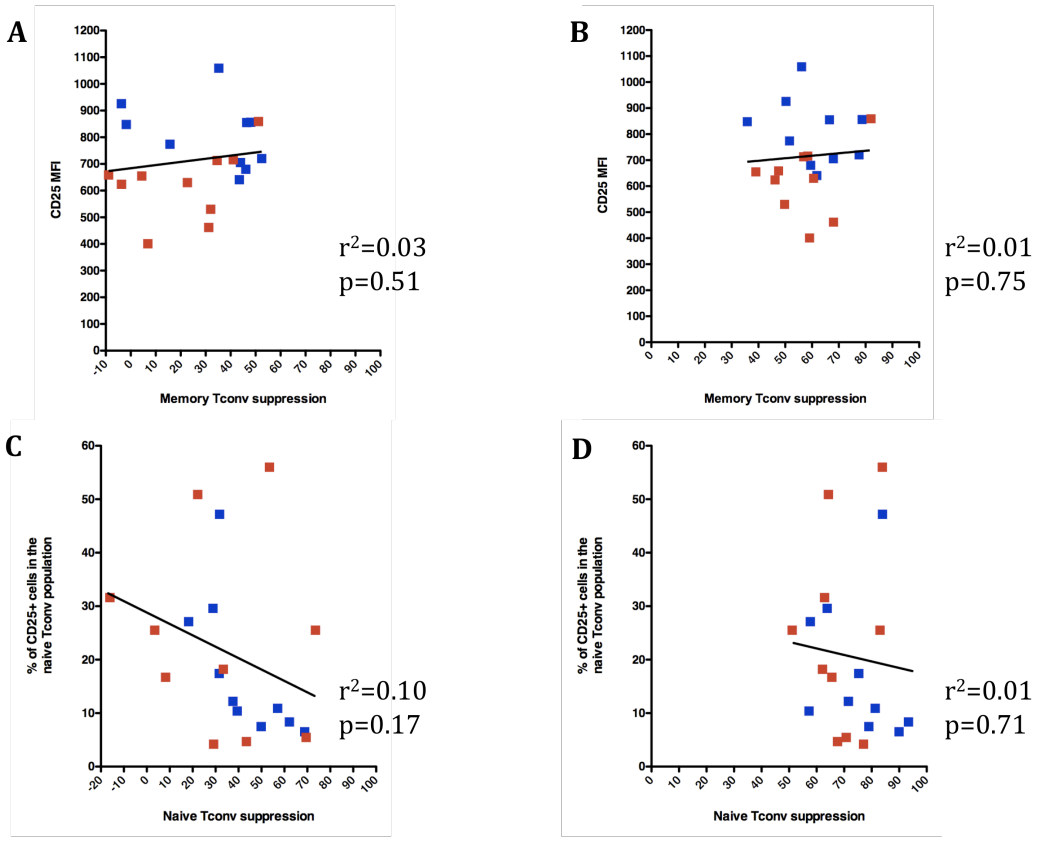

Figure 5.22 Correlation of CD25 with Tconv suppression

The correlation between memory Tconv CD25 expression and the mean level of suppression of these cells in autologous co-cultures, when stimulated with beads at 1:1 (figure A) and 1:2.5 (Figure B) was examined. Also the percentage of CD25+ naïve Tconv and suppression of naïve Tconv in autologous co-cultures, when stimulated with beads at 1:1 (figure C) and 1:2.5 (Figure D) is shown. All 20 donors are shown. Blue squares represent protected donors whilst red squares represent susceptible donors. Correlations were assessed by linear regression with p values showing how significantly non-zero the slope was.

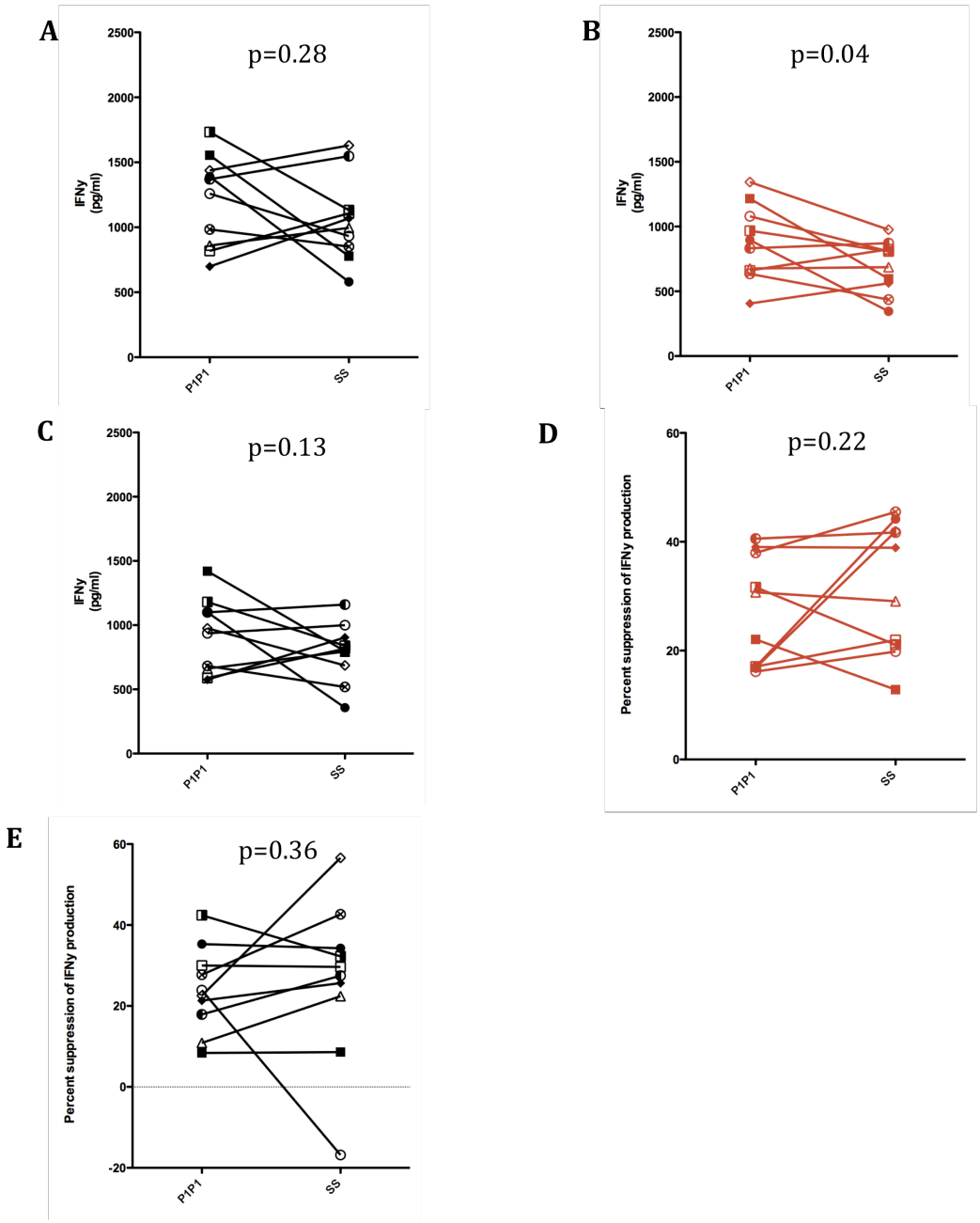

Figure 5.23 IFNγ production and suppression by CD4+ memory Tconv alone or in co-culture with either autologous or Standard Tregs

The level of IFNγ produced (pg/ml) by memory Tconv alone (Figure A) or in co-culture with autologous Tregs (Figure B) or Standard Tregs (Figure C) is shown. The percent suppression of IFNγ in autologous co-cultures (Figure D)

and Standard Treg co-cultures (Figure E) was also calculated using the formula: % suppression= 100-(pg/ml of IFNγ in the presence of Tregs ÷ pg/ml of IFNγ in the absence of Tregs) x 100). The dotted line is placed at zero on the y-axis. Data from Figures A and D was analysed by a one-tailed Wilcoxon matched-pairs signed rank test, whilst data from Figures B, C and E was analysed by a one-tailed paired t test.

or Standard Treg co-cultures. Naïve Tconv produced much lower levels of IFNγ and no significant differences were seen between donor groups in either the level of production or suppression of this cytokine (data not shown).

There were no significant differences between protected and susceptible donors in the level of IL-10 produced by memory Tconv either alone or in co-culture with autologous or Standard Tregs (Figure 5.24). Very little IL-10 was produced by naïve Tconv in the absence of Tregs and although levels did increase when in co-culture with Tregs, no significant differences were seen between protected and susceptible donors (data not shown). The IFNγ:IL-10 ratios were also examined for memory Tconv cultures (Figure 5.25). The only significant difference in cytokine production has been the higher level of secretion of IFNγ by memory Tconv in culture with autologous Tregs from protected donors compared to susceptibles. However the IFNγ:IL-10 ratios suggest that more protected donors produced lower levels of IFNγ in relation to IL-10 in autologous co-cultures, compared to susceptible donors. No apparent significant difference between donor groups was evident in Standard Treg co-cultures.

5.3 Discussion

The purpose of this chapter was to test the hypothesis that the Protective P1 haplotype (due to the presence of protective alleles at SNP.495 and

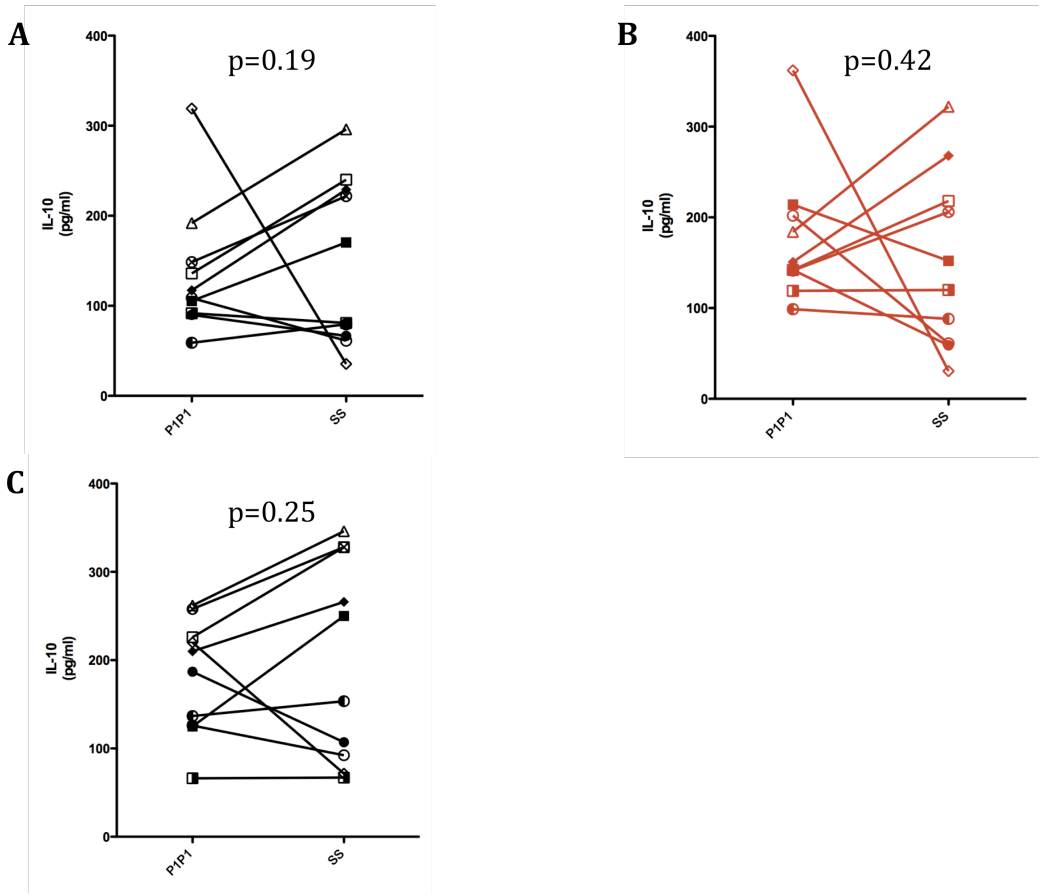

Figure 5.24 Production of IL-10 by CD4+ memory Tconv alone or in co-culture with either autologous or Standard Tregs

The level of IL-10 (pg/ml) produced by memory Tconv alone (Figure A) or in co-culture with autologous Tregs (Figure B) or Standard Tregs (Figure C). Protected donors (P1P1) were compared with susceptible (SS) donors. Data from Figures A and B was analysed by a one-tailed Wilcoxon matched-pairs signed rank test, whilst data from Figure C was analysed by a one-tailed paired t test.

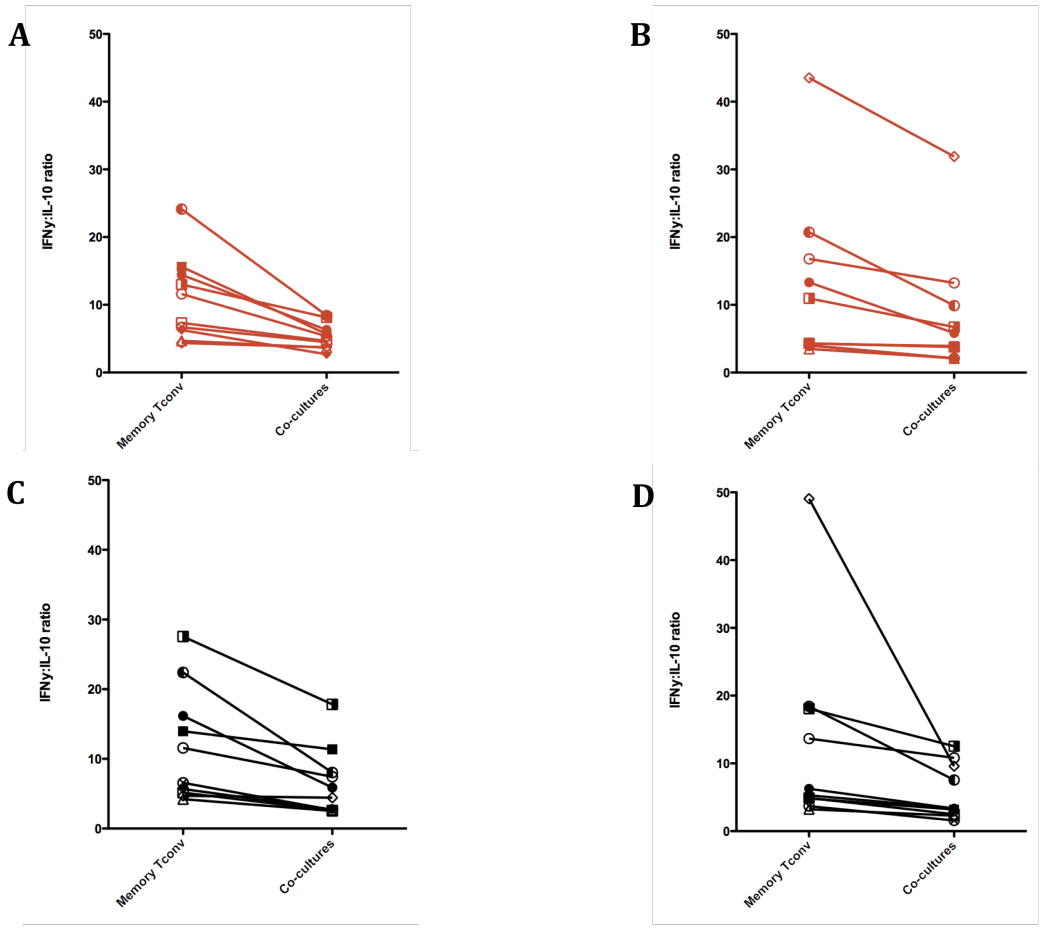

Figure 5.25 IFNγ:IL-10 ratios for memory Tconv cultured alone compared with ratios from autologous or Standard Treg co-cultures

The IFNy:IL-10 ratios for cultures of memory Tconv alone compared to autologous co-cultures are shown for protected donors (Figure A) and susceptible donors (Figure B). The IFNy:IL-10 ratios for cultures of memory Tconv alone compared to Standard Treg co-cultures are also shown for protected donors (Figure C) and susceptible donors (Figure D).

SNP.286) influences the ability of Tregs to suppress Tconv proliferation.

5.3.1 Possible implications of the Protective P1 haplotype on Treg function

The Protective P1 haplotype has protective alleles at SNP.495 and SNP.286, which are associated with increased memory Tconv CD25 expression, increased memory Tconv IL-2 production and lower percentages of CD25+ naïve Tconv, respectively (Dendrou *et al.*, 2009). As Tregs require IL-2 for maintenance and suppressive function (Taams *et al.*, 2001, Zorn *et al.*, 2006) the implications of this haplotype on Treg function compared to the Fully Susceptible haplotype, were tested. Also as postulated in Chapter Four (Section 4.1) the percentage of CD25+ naïve Tconv may be evidence of a mechanism to prevent naïve Tconv from fully differentiating into memory Tconv and the lower percentage of these cells associated with the protective allele at SNP.286, may represent a higher number of naïve Tconv differentiating into memory Tconv and therefore producing higher levels of IL-2.

The study conducted in Chapter Four on the implications of the protective allele at SNP.286 on Treg function demonstrated the importance of selecting donors both for the genotype and representative immunophenotype of interest, when examining small groups of donors (Chapter Four, Section 4.3.1). Therefore the Cambridge BioResource was utilised for the study in

this chapter to recruit and select pairs of protected and susceptible donors. However whilst the extremes of the immunophenotype associated with the allele at SNP.495 were selected for, the immunophenotype associated with the allele at SNP.286 was not. Analysis of the percentages of CD25+ naïve Tconv between pairs revealed no significant difference between protected and susceptible donors. This hindered the ability to examine the implications of the allele at SNP.286 on Treg maintenance and ability to suppress.

5.3.2 Analysis of CD25 expression by Tconv and Treg populations

The extremes of memory Tconv CD25 expression were selected for in the pairs of donors examined in this study, as shown by analysis of CD25 implemented as part of the pSTAT5a assay and on data gathered during the FACS isolation (although the latter showed a lower level of significance). Previous work by our collaborators examined levels of CD25 on Tregs and found donors with the protective allele at SNP.495 had higher levels of CD25 on CD4+ FOXP3+ T cells, although this difference was not significant (Dendrou *et al.*, 2009). The data in this chapter conducted as part of the pSTAT5a assay found Tregs from individuals with the protective allele to have significantly higher levels of CD25 than those with a susceptible allele. However, data obtained during the FACS isolation did not reveal a difference in CD25 expression on Tregs was seen between protected and susceptible donors. Following the completion of the work in this chapter, an assay was

developed in this laboratory by Dr. Jennie Yang, in which FOXP3 and pSTAT5a could be measured simultaneously in T cells in whole blood. This provided the opportunity to re-examine these results. Dr. Jennie Yang examined a new cohort of donors consisting of six of the original pairs and seven new pairs (Figure 5.26). Comparison of Treg subpopulations demonstrated that mTregs and aTregs from protected donors showed significantly higher levels of CD25. No significant difference in the expression of rTregs was seen between groups of donors (Figure 5.26). (CD25 on the total Treg population was not examined (personal communication-(Yang, 2013)). The reasons for these seemingly contradictory findings on Tregs may be due to the processes used. Analysis of cell populations in the pSTAT5a assay and the whole blood assay involves the fixation of cells prior to staining, which may influence the ability with which the anti-CD25 antibody binds. Furthermore, cells were stained with a different cocktail of antibodies in these assays compared to those which were isolated by FACS and were subsequently analysed using different flow cytometers (FACS Canto II flow cytometer instead of a FACS Aria cell sorter). Also the analysis of CD25 expression by our collaborators, which identified a trend, involved fixation of the cells following staining (Dendrou *et al.*, 2009).

Analysis of pSTAT5a by the whole blood assay revealed aTregs from protected donors had significantly higher levels of pSTAT5a than susceptible donors at low levels of IL-2 (data not shown). However, at high IL-2 doses, no significant differences between the donor groups were seen.

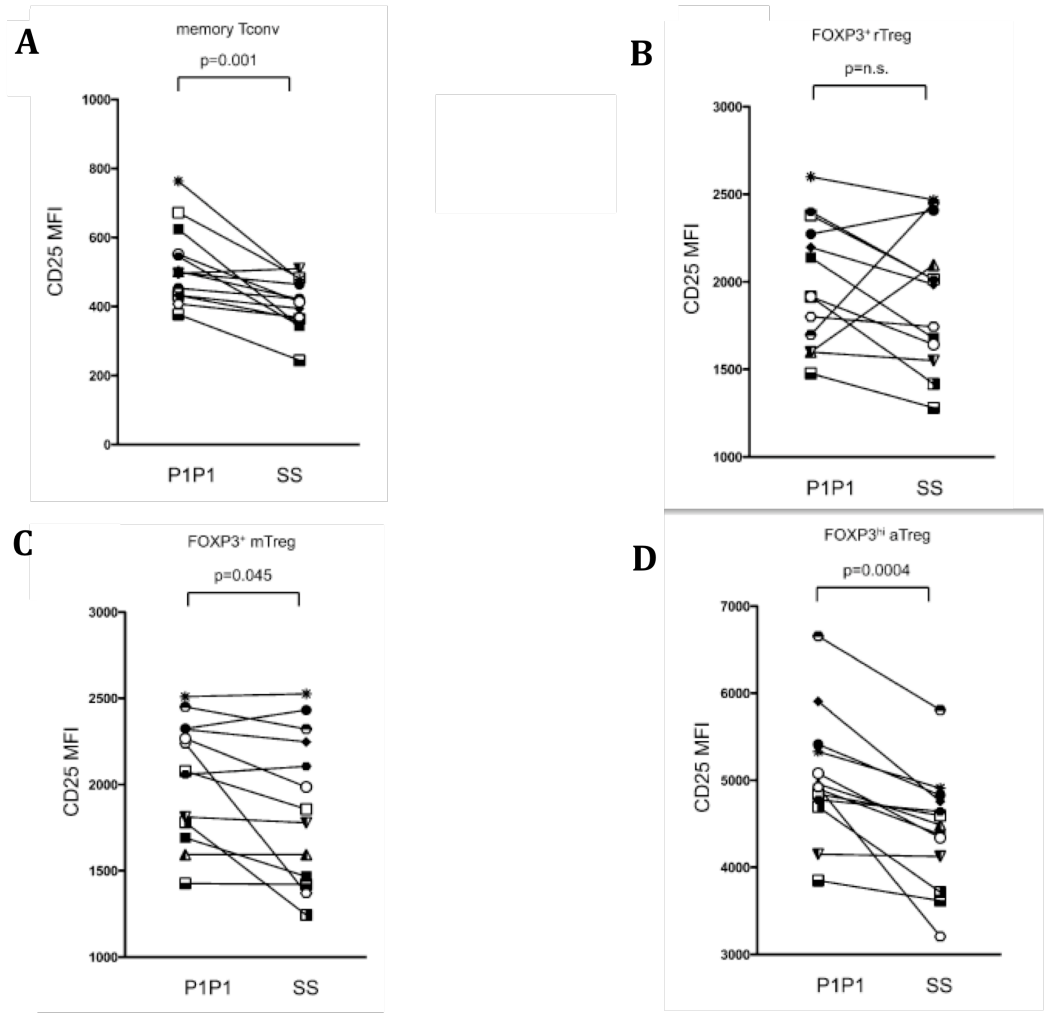

Figure 5.26 FOXP3/pSTAT5a staining conducted in whole blood

The pSTAT5a study was repeated using a technique optimised by Dr. Jennie Yang, using whole blood. CD25 (MFI) expression of memory Tconv (Figure A), FOXP3+ rTreg (Figure B), FOXP3+ mTreg (Figure C) and FOXP3hi aTreg (Figure D) is shown. Protected donors (P1P1) were compared with susceptible (SS) donors. Data provided courtesy of Dr. Jennie Yang. Data was analysed by a two-tailed paired t test.

It could be speculated that these were saturating doses of IL-2. Which may indicate the difference seen at lower levels is suggestive of a defective response to IL-2 but at saturating doses, there is no defect in the ability of STAT5a to undergo phosphorylation. No significant difference in the levels of pSTAT5a was seen in rTregs or mTregs between protected and susceptible donors. rTregs from both protected and susceptible donors showed comparable levels of CD25 on these cells, perhaps explaining this. The lack of a significant difference in pSTAT5a in mTregs expression between protected and susceptible donors may be due to other factors affecting the IL-2 signalling pathway.

5.3.3 The protective allele at SNP.495 is associated with increased Treg maintenance and function

Prior work by our collaborators, together with the results in this chapter, including the findings by Dr. Jennie Yang, demonstrate that protected individuals have higher levels of CD25 on memory Tconv (Dendrou et al., 2009), total Tregs and the mTreg and aTreg subpopulations compared to susceptible individuals. Therefore, these cell populations are able to respond more readily to IL-2 thus producing more pSTAT5a (although not in the case of mTregs).

Due to an increased response to IL-2, Helios+ Tregs (nTregs) from protected donors show higher levels of FOXP3 expression than susceptible donors, a

transcription factor demonstrated to be responsible for the suppressive function of Tregs (Fontenot *et al.*, 2003, Bacchetta *et al.*, 2006, Wildin *et al.*, 2001) (Chapter One, Section 1.2.3). nTregs were selected due to their expression of the transcription factor Helios, thought to be a marker for these cells (Thornton *et al.*, 2010) (although it should be noted this is controversial (Chapter One, Section 1.2.4)). Finally suppression of memory Tconv at the highest stimuli strength was higher in the protected donors. As discussed earlier, the signal strength is important in identifying differences between groups of individuals (Chapter Three, Section 3.3.2).

5.3.4 Conclusions

The findings in this chapter suggest that memory Tconv from protected donors may be better at maintaining Tregs and supporting their suppressive function. A model demonstrating how the immunophenotypes of these cells act synergistically to affect the level of suppression is shown in Figure 5.27. In protected donors the increase in IL-2 production by memory Tconv and the higher level of CD25 on aTregs directly leads to increased signalling and maintenance of FOXP3 which results in increased suppression of memory Tconv. However, it should be noted that many genes functioning within the IL-2 signalling pathway are associated with T1D, and may also be implicated in this model.

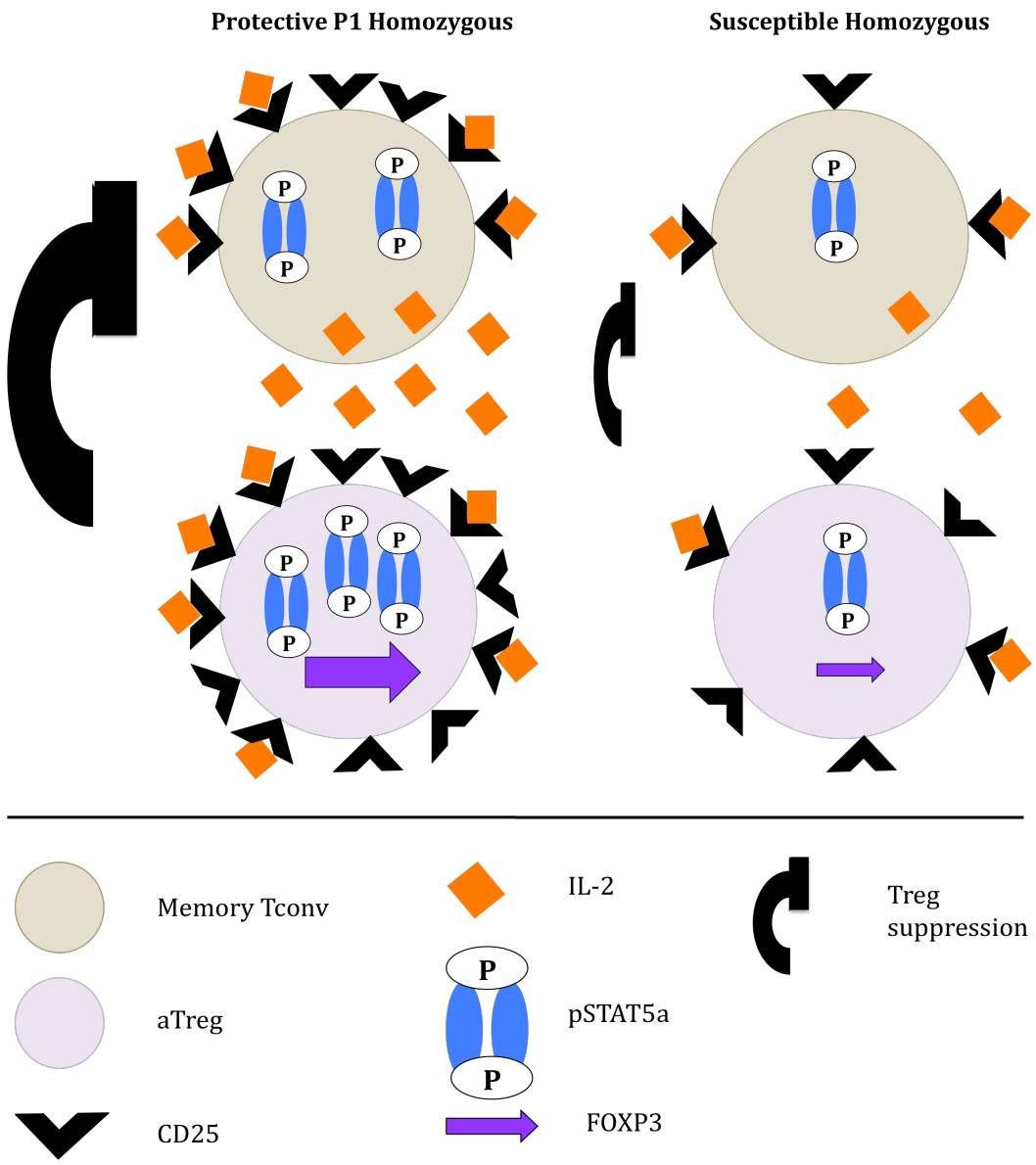

Figure 5.27 Illustration of the ensuing implications of the allele at SNP.495

The protective allele at SNP.495 is associated with higher memory Tconv CD25 expression and IL-2 production and higher CD25 expression on aTregs. This results in higher pSTAT5a expression in both cell types and higher FOXP3 expression in aTregs, allowing these cells to suppress memory Tconv to a higher degree, compared to those with the susceptible allele.

Chapter Six: Comparison of Treg function within pairs of type 1 diabetes-discordant monozygotic twins

6.1 Introduction

6.1.1 Monozygotic twin studies

T1D is a polygenic disease with over fifty genes so far associated with predisposition to this disorder (Eisenbarth, 2009, Todd, 2010). However, many human traits are thought to be the product of both nature and nurture and T1D is no exception. It is hypothesised that the presence of genetic predisposition together with exposure to an, as yet unknown, environmental trigger(s) leads to T1D pathogenesis (Knip *et al.*, 2005) (Chapter One, Section 1.13). The appreciation for the interplay between genetic and environmental factors in T1D has come largely from studies of monozygotic twins, who are genetically identical to each other. Concordance rates of 13-55% have been reported for T1D in these twins (Kaprio *et al.*, 1992, Olmos *et al.*, 1988, Redondo *et al.*, 2001, Barnett *et al.*, 1981) demonstrating the importance of genetic factors.

However, it must be remembered that as well as sharing their DNA, monozygotic twins have usually shared the same environment, especially in childhood, when T1D is typically diagnosed (Atkinson and Eisenbarth, 2001). In comparison to non-twin siblings, the concordance rate of whom is

around 2% (Redondo *et al.*, 1999), monozygotic twins will have shared the same *in utero* and early post-natal environment (Redondo *et al.*, 2004). However, dizygotic twins, who share the same amount of their genes as non-twin siblings have recorded concordance rates of 0-13% (Redondo *et al.*, 1999, Kumar *et al.*, 1993). Whilst it is surprising that Redondo *et al.* (1999) reported 0% concordance among dizygotic twins, these twin subjects were fewer in number and were observed for a much shorter period of time than monozygotic twins, possibly explaining this result. However, the collective results of studies on dizygotic twins demonstrate the high concordance rate of monozygotic twins cannot be fully explained by shared environment alone. Although they are genetically identical, monozygotic twins can differ from each other in terms of epigenetics, somatic mutations and in the case of females, random X-chromosome inactivation (Salvetti *et al.*, 2000).

6.1.2 Predicting T1D-concordance in monozygotic twins

Diagnosis of the index twin in early childhood places the co-twin at high risk of also developing this disease, compared to those whose twins were diagnosed as adults (Redondo *et al.*, 2001, Johnston *et al.*, 1983, Olmos *et al.*, 1988, Millward *et al.*, 1986). This is particularly true within the first five years following diagnosis. However, after eleven years of disease discordance, the risk decreases to 3% (Millward *et al.*, 1986). An example of how the rate of concordance decreases over time is shown in Figure 6.1 and Table 6.1. Figure 6.1 also clearly demonstrates that co-twins

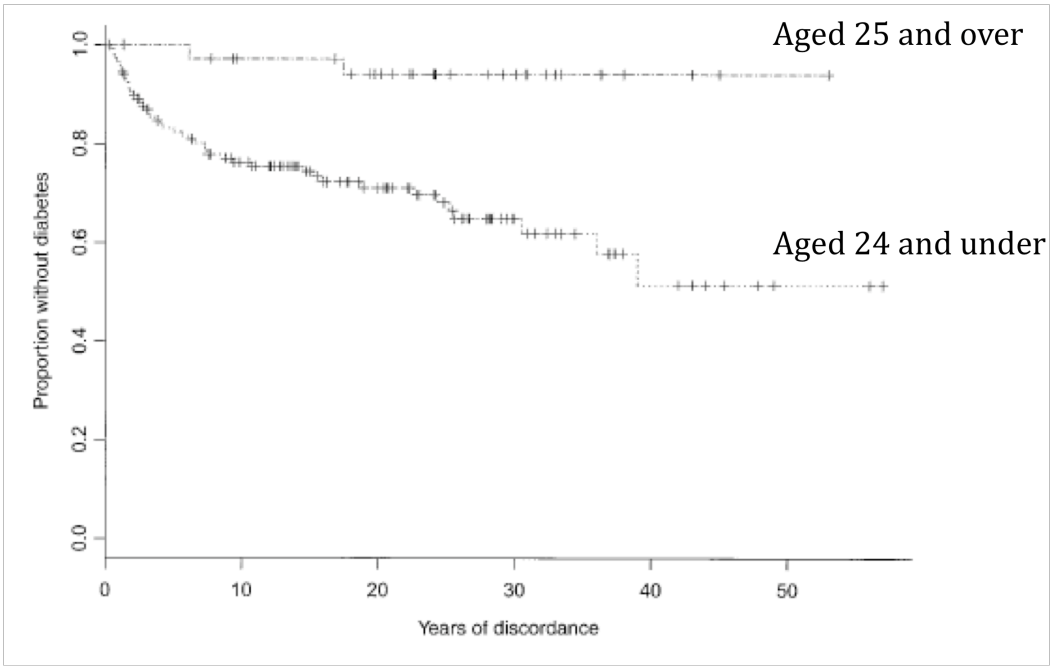

Figure 6.1 Diagnosis of the index twin before the age of twenty-five places the co-twins at higher risk of developing T1D

The graph shows the proportion of non-diabetic co-twins remaining diabetes-free over the course of more than fifty years. The data was compiled from a cohort of over 180 T1D-discordant monozygotic twins in both Great Britain and the U.S.A. The dotted line (….) represents the co-twins of index twins diagnosed aged twenty-four and under, whilst the dot-dashed line (._._.) represents the co-twins of index twins diagnosed aged twenty-five and over. Cross-lines represent twins who have developed T1D. The progression to T1D in co-twins of those diagnosed aged twenty-five and over was significantly lower than those diagnosed aged twenty-four and under (p=0.004). Data reproduced from Redondo et al. (2001).

Relationship to diabetic patient	Factor	Risk	References
Sibling		2%	(Redondo *et al.*, 1999)
Dizygotic twin		0-13%	(Redondo *et al.*, 1999, Kumar *et al.*, 1993)
Monozygotic twin		13-55%	(Barnett *et al.*, 1981, Kaprio *et al.*, 1992, Olmos *et al.*, 1988, Redondo *et al.*, 2001)
	-30 years of discordance. -Diabetic twin diagnosed <24 years of age	38%	(Redondo *et al.*, 2001)
	-30 years of discordance. -Diabetic twin diagnosed >24 years of age	6%	(Redondo *et al.*, 2001)
	-20 years of discordance. -Diabetic twin diagnosed <20 years of age. -No islet auto-antibodies in non-diabetic twin	<2%	(Beyan *et al.*, 2010)

Table 6.1 Summary of the risk of developing T1D

This simplified table shows the calculated risk of an individual developing T1D, according to their relationship to the diabetic patient and the influence of other confounding factors.

whose index twins were diagnosed before the age of twenty-four show higher rates of concordance than those whose twins developed T1D at twenty-five and over. Another indicator is the presence of islet autoantibodies in the blood of the co-twin, such as GADA and IA-2 antibody (Johnston *et al.*, 1989). Having more than one islet autoantibody, a high antibody titre and displaying persistence of antibody over time, all add to the risk of disease.

6.1.3 Defective Treg function in T1D may be genetically-determined

As discussed in Chapter One, Section 1.14, several studies in T1D have demonstrated significantly reduced suppressive function of Tregs in diabetics compared to control individuals (Lindley *et al.*, 2005, Lawson *et al.*, 2008, Brusko *et al.*, 2005, Jana *et al.*, 2010, Glisic-Milosavljevic *et al.*, 2007b). It has also been shown that Treg function is significantly decreased in islet-autoantibody-positive individuals compared to controls (Jana *et al.*, 2010, Glisic-Milosavljevic *et al.*, 2007b). Although in the study by Jana *et al* (2010) these donors still exhibited higher levels of suppression compared to ROT1D donors. However, it is not known whether the defective Treg function shown in these studies is genetically determined or a consequence of the disease itself. Many of the genetic polymorphisms thus far associated with T1D operate within the immune system (Eisenbarth, 2009, Todd, 2010) and a considerable number may concern Treg function, *i.e. IL-2RA, PTPN2, PTPN22, CTLA-4* and *IL-2* (Chapter One, Section 1.13). Buckner and

colleagues have previously shown a reduced response to IL-2 by Tregs from diabetics compared to controls and implicated a role for the negative regulator of this signalling pathway, PTPN2 (Long *et al.*, 2010). Also, work from Chapter Five suggests an *IL-2RA* SNP associated with T1D, SNP.495, is linked with decreased Treg maintenance and function.

The purpose of this section of the thesis was to test the hypothesis that non-diabetic co-twins have comparably poor levels of Treg suppression to their index twins. Treg function from four pairs of T1D-discordant twins and six healthy control donors was examined. As in the other studies of this thesis, the cells of interest were isolated by FACS (as described in Chapter Two, Section 2.8.4 and Chapter Three, Section 3.2.1) and proliferation and suppression of the CD4+ Tconv population were measured by either two-cell Treg suppression assays activated by Dynabeads® (Chapter Two, Section 2.8.8 and Chapter Three, Section 3.2.2) or three-cell Treg suppression assays activated with plate-bound anti-CD3 antibody with soluble anti-CD28 antibody and irradiated ACs (Chapter Two, Section 2.8.8 and Section 3.2.5). In addition to co-culturing the Tconv with autologous Tregs, crossover co-cultures were set-up using Tregs from the opposite twin, to compare resistance of the Tconv and the suppressive function of the Treg population (Chapter Two, Section 2.8.8 and Chapter Three, Section 3.1.1). Statistical analyses were performed as described in Chapter Two, Section 2.8.11 and are given in each figure. A summary of all procedures conducted is shown in Figure 6.2.

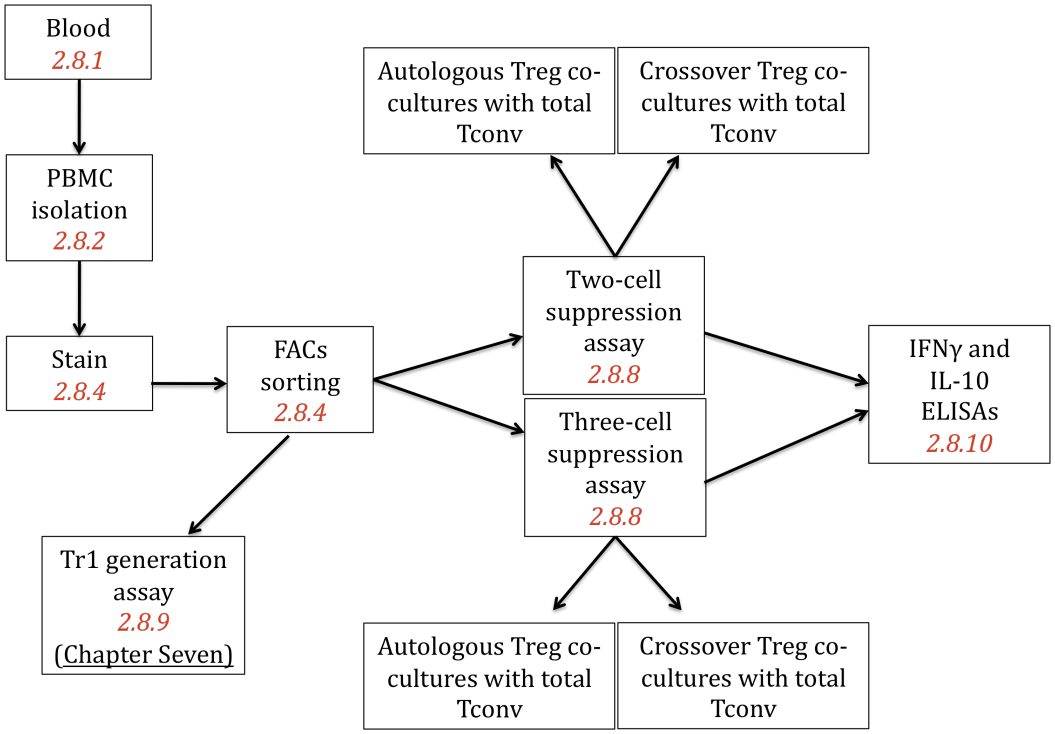

Figure 6.2 Flow chart summarising the procedures conducted on each sample

The above flow chart demonstrates the order and number of all procedures conducted on each pair of samples. The red italics show the section in Chapter Two giving full details of protocols used.

6.2 Results

6.2.1 Donor selection

To compare Treg function between diabetics and their non-diabetic co-twins, four pairs of monozygotic twins discordant for T1D were examined in this study. It was the intention to recruit a minimum of ten sets of twins (see Section 6.3.1), however due to a series of unforeseeable circumstances this was not possible in the time given. The twins were selected from the British Diabetic Twin Study cohort, which currently consists of 546 twin pairs (both monozygotic and dizygotic twins) (Beyan *et al.*, 2012). Of these, around fifty were suitable for this study (personal communication-(Leslie, 2012)). The study in this chapter culminates in the Treg suppression assay, which measures the level of suppression of Tconv proliferation after six days in co-culture with Tregs (Chapter Three, Sections 3.2.2-3.2.7). As such low numbers of fresh cells are used in this assay (a maximum of $2.5x10^3$) and cells are cultured over several days, one of the limitations of this assay is its potential vulnerability to day-to-day variations. In an attempt to reduce these variations, a number of measures were taken in the processing of the samples to keep all factors as constant as possible. As part of this, both twins were bled on the same day, at the same time as each other or at least within a narrow window of time. As the twins analysed were adults, it was often the case that twins were living in different locations to each other. The phlebotomists working with collaborators at Queen Mary University of London had the task of arranging for it to be possible for the twins to be bled

in the same place or strategising journeys to bleed twins within a specified time-frame; a task hindered by the sudden departure of the senior phlebotomist for a post overseas. Also, previous studies conducted by the collaborators generally did not require blood to be taken from both twins on the same day, therefore new strategies had to be implemented to achieve this.

The twin pairs consisted of two males and two females with a mean age of 43.5 years S.D. ±12.4. The data on the twin donors is summarised in Table 6.2. These twins were selected according to indicators the co-twins were at very low risk of ever developing T1D (≤ 2%) (Beyan *et al.*, 2010) (see Section 6.1.2). Although the index twins were diagnosed in childhood (mean age at diagnosis 13.0 S.D. ±5.48) this ensured that a long period of discordance had elapsed (mean disease duration 30.3 years S.D. ±9.0) and the non-diabetic co-twins had always tested negative for GADA and IA-2 antibody (Table 6.3). Whilst none of the index twins possessed IA-2 autoantibodies and only two were positive for GADA, it should be noted that autoantibody sampling did not take place at diagnosis, but a mean of 7.5 years S.D. ±4.66 years later. Studies suggest that most autoantibodies decline over time in LST1D patients (Jaeger *et al.*, 1997, Rakyan *et al.*, 2011).

As a comparison, Treg function was also assessed in control individuals; recruited from the local cohort of donors. It was also intended for ten

Twin pair no.	Symbol	Gender	Age	DRB1 Genotype
1		F	45	DR3 DR4
2		M	25	DR4
3		F	47	DR4 DR6
4		M	57	DR1 DR3

Table 6.2 Details of the monozygotic twin pair donors recruited for the study examining Treg function in T1D-discordant monozygotic twins

M=Male, F=Female

Twin pair no.	Diabetic twin				Non-diabetic twin	
	Age at diagnosis	Years since diagnosis	GADA	IA2	GADA	IA2
1	14	31.0	-	-	-	-
2	7	17.4	+	-	-	-
3	11	35.9	+	-	-	-
4	20	37.0	-	-	-	-

Table 6.3 Age at diagnosis, disease discordance and presence of islet autoantigens in monozygotic twins

control donors to be examined, equally interspersed throughout this study. As controls were analysed on different days to the monozygotic twins, the study was designed as such to reduce any bias arising from day-to-day variations. However, as the complete cohort of twin donors could not be processed, only six control donors were examined. The controls were gender- and age-matched to the twin pairs (three males, three females, with a mean age of 41.67 years S.D. ±11.24). The data on the control donors is summarised in Table 6.4. Also, the control donors were matched as far as possible, according to the HLA-DRB1 genotype, although no controls had the DR3/DR4 heterozygous genotype, whereas one twin pair did. However, this genotype has been reported by others conducting similar studies to be rare in the non-diabetic population (Kallmann *et al.*, 1999, Johnston *et al.*, 1983).

6.2.2 Isolation of the cells

As described in Chapter Two, Section 2.8.4 and Chapter Three, Section 3.2.1, FACS was used to isolate total CD4+ CD25hi CD127lo Tregs, total CD4+ CD25-/+ Tconv and a mixture of CD14+ monocytes and CD19+ B cells from PBMC from each donor (Figure 6.3). The monocytes and B cells were then irradiated and used in the subsequent Treg suppression assays as ACs.

6.2.3 Analysis of the total population and subpopulations of Tregs

Several studies have demonstrated there is no difference in the frequency of Tregs in the peripheral blood of diabetics compared to controls

Control donor no.	Gender	Age	DRB1 genotype
T001	F	43	DR1 DR4
T030	F	33	DR3
T033	M	37	DR1 DR4
T070	F	44	DR1 DR4
T073	M	31	DR3
T079	M	62	DR4

Table 6.4 Details of the control donors recruited for the study examining Treg function in T1D-discordant monozygotic twins

M=Male, F=Female

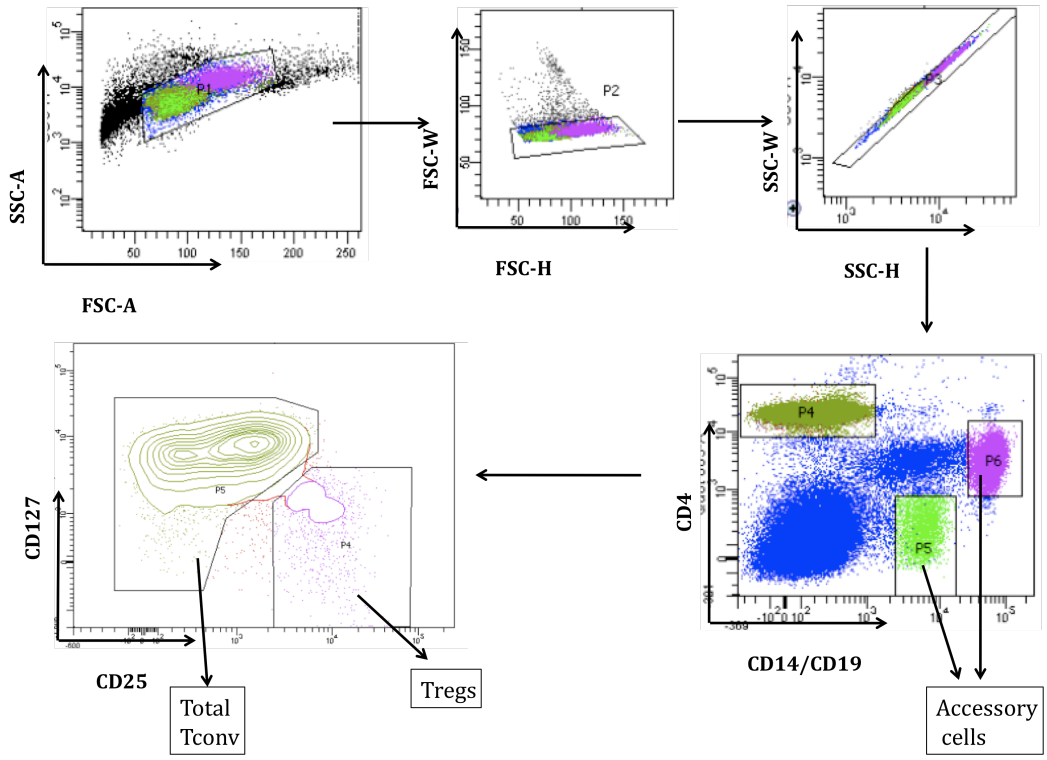

Figure 6.3 Gating strategy for the isolation of Tregs, total CD4+ Tconv, monocytes and B cells

The gating used to isolate cells for the twin study is shown. Firstly, lymphocytes were gated and doublets removed. After gating on CD4+ T cells, the Tconv were isolated by high expression of CD127 and negative to intermediate expression of CD25. Tregs were isolated according to high expression of CD25 correlating with low levels of CD127. Monocytes and B cells were isolated together according to cells that were CD4+ CD14+ and CD4- CD19+, respectively.

(Lindley *et al.*, 2005, Lawson *et al.*, 2008, Brusko *et al.*, 2005, Putnam *et al.*, 2005, Liu *et al.*, 2006b, Ferraro *et al.*, 2011) (Chapter One, Section 1.14). To examine whether there was a difference between the twin pairs and the controls in this study, the percentage of total Tregs was compared (Figure 6.4). From each donor, a separate sample of PBMC was stained with the same antibody panel used to isolate the cells plus an additional anti-CD45RA antibody. This enabled the rTreg, mTreg and aTreg subpopulations to also be examined (see Chapter One, Section 1.3 and Figure 4.4B) (Figure 6.4). No difference in any of these Treg subpopulations was observed across the three donor sets. However, pair-wise analyses between twins showed diabetic twins had significantly higher levels of total Tregs compared to their co-twins (Figure 6.5).

6.2.4 Analysis of the Tconv population

Although the total CD4+ Tconv population was sorted, the proportion of memory and naïve Tconv were measured. Others have shown memory CD4+ Tconv are more resistant to Treg suppression than naïve CD4+ Tconv (Afzali *et al.*, 2011, Jana *et al.*, 2010) which could have implications on the suppression assay. The percentage of these two cell populations and the memory:naïve Tconv ratio was compared between the three donor groups but no differences were seen (Figure 6.6). There were also no differences in the level of CD25 expressed by these cells (data not shown).

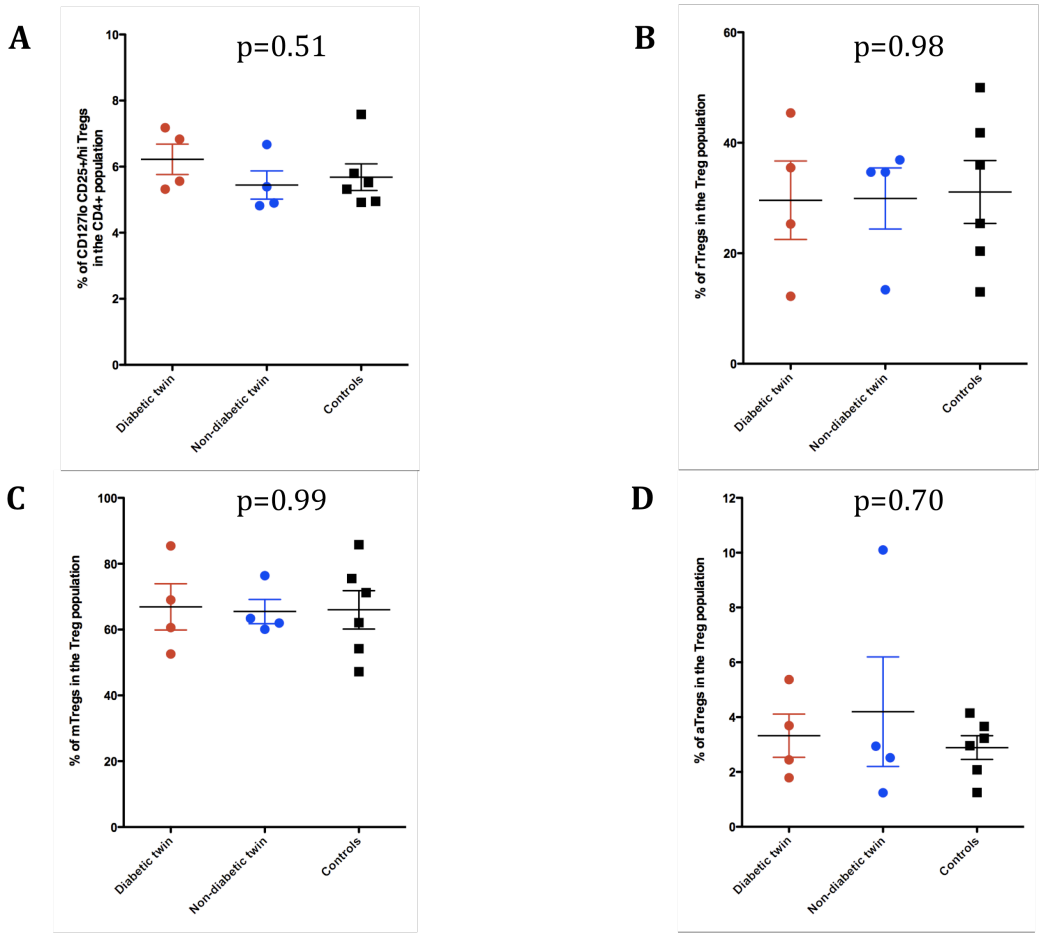

Figure 6.4 Percentages of total and subpopulations of Tregs

The percentage of total CD4+ CD25hi CD127lo Tregs in the CD4+ T cell population (Figure A) and percentage of rTregs (Figure B), mTregs (Figure C) aTregs (Figure D) among total Tregs, was measured for all donors. Red circles represent diabetic twins, blue circles represent non-diabetic twins and the black squares denote control donors. Data was analysed by a one-way ANOVA.

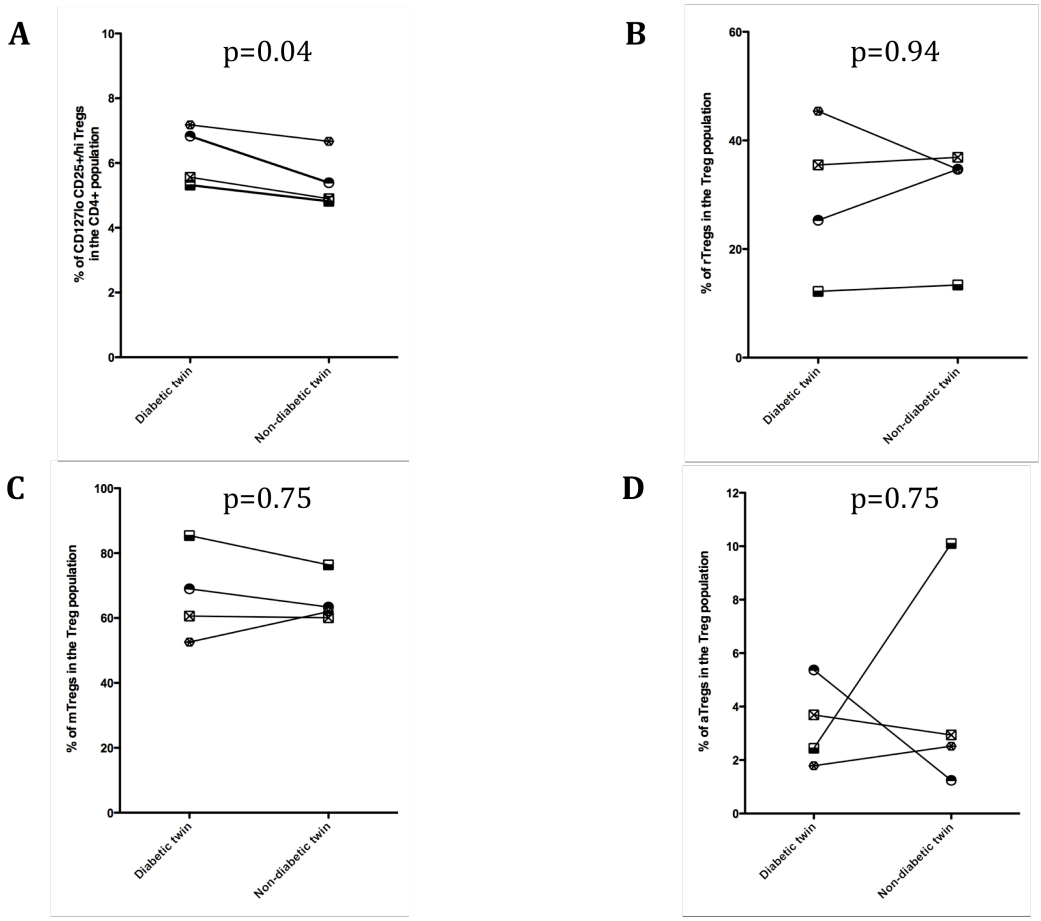

Figure 6.5 Twin pair-wise analyses of percentages of total and subpopulations of Tregs

The percentage of total CD4+ CD25hi CD127lo Tregs in the CD4+ T cell population (Figure A) and percentage of rTregs (Figure B), mTregs (Figure C) aTregs (Figure D) among total Tregs, was compared in pairs of twins. Data was analysed by a two-tailed paired t test.

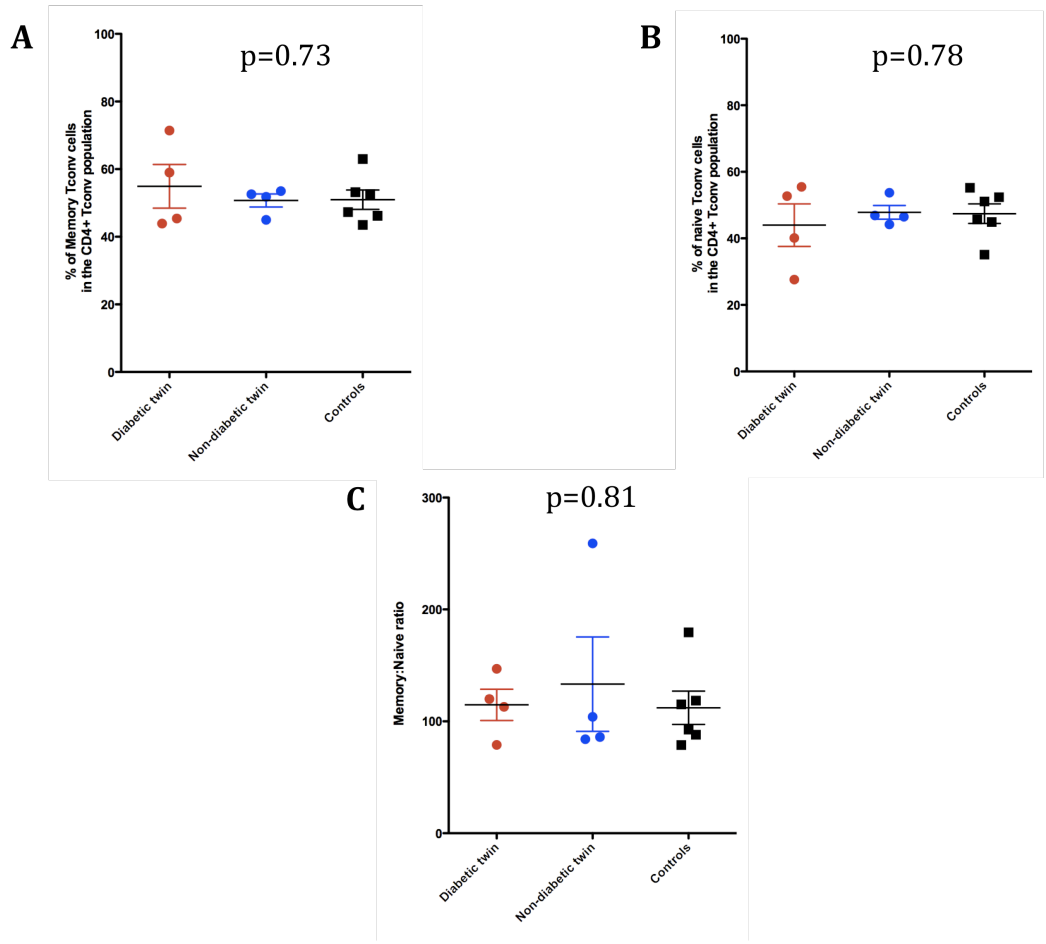

Figure 6.6 Percentages of memory and naïve Tconv

Percentage of memory Tconv amongst CD4+ Tconv (Figure A), percentage of naïve Tconv amongst CD4+ Tconv (Figure B) and the memory:naïve Tconv ratio (Figure C). Red circles represent diabetic twins, blue circles represent non-diabetic twins and the black squares denote control donors. Data was analysed by a one-way ANOVA.

6.2.5 Analysis of the accessory cell population

Another factor, which may have implications on the suppression assay, are the ACs used. Autologous monocytes and B cells were added to suppression assays stimulated with plate-bound anti-CD3 antibody. However, monocytes are suggested to act as superior APC compared to B cells (Beck *et al.*, 1996). Therefore, the proportion of monocytes and B cells were compared across all donors. There were no differences in the percentage of monocytes or B cells of PBMC either between all sets of donors or between pairs of twins (data not shown). However, as a mixture of these cells was added to cultures, the ratio of monocytes to B cells was compared between donors (Figure 6.7). No difference in this ratio was found when all three donor sets were compared. However, twin pair-wise analysis revealed that three out of four diabetic twins had higher monocyte:B cell ratios than their co-twins; a difference which almost reached significance.

6.2.6 Proliferation of the CD4+ Tconv population

The Treg suppression assays were stimulated with either plate-bound anti-CD3 antibody at 0.25μg/ml and 0.5μg/ml (three-cell suppression assay) or Dynabeads® at bead:cell ratios of 1:1 and 1:2.5 (two-cell suppression assay). The level of Tconv proliferation in the absence of Tregs was examined in response to each stimulus, as this could clearly impact on the level of suppression seen. Unfortunately, due to a lack of cells, the suppression assays stimulated with the two Dynabeads® concentrations could not be set

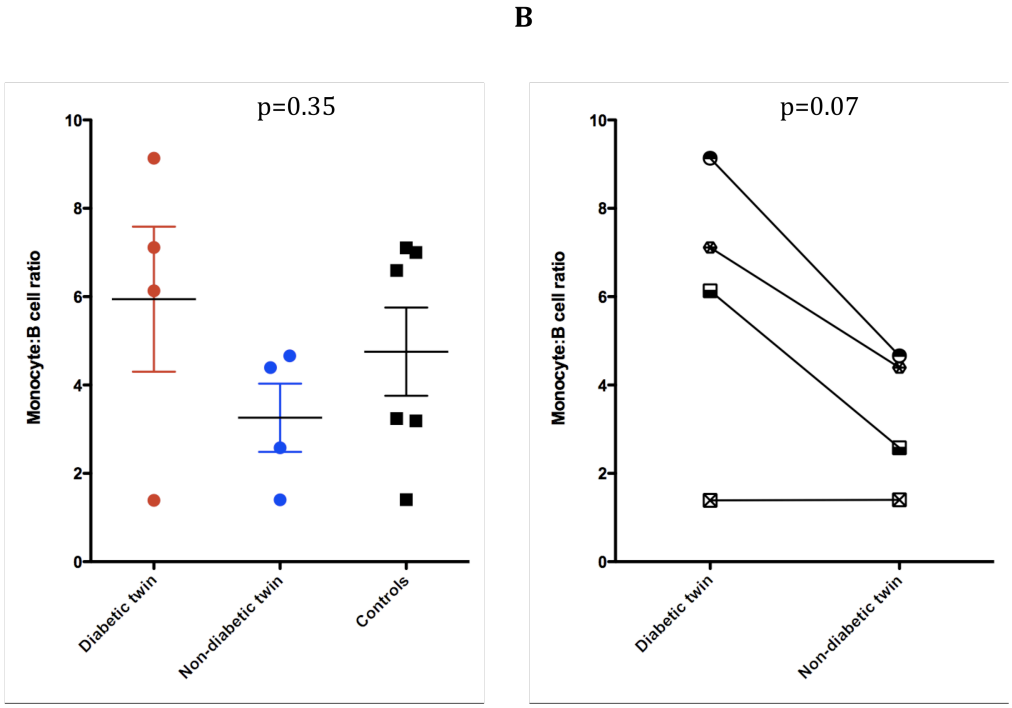

Figure 6.7 Analysis of the accessory cell population

The ratio of monocytes to B cells was measured for all donors (Figure A). Red circles represent diabetic twins, blue circles represent non-diabetic twins and the black squares denote control donors. Pair-wise analysis of the monocyte:B cell ratio is shown for twin pairs (Figure B). Data in Figure A analysed by a one-way ANOVA whilst the data Figure B was analysed by a two-tailed paired t test.

up for twin-pair two. There was no difference in the level of proliferation when cultures were stimulated with plate-bound anti-CD3 antibody (Figure 6.8). Although Tconv from all three sets of donors proliferated to the same extent when stimulated with Dynabeads®, analysis of just the twin pairs showed the diabetic donors proliferated to a significantly higher degree than their co-twins, when stimulated with beads at 1:2.5 (Figure 6.9).

6.2.7 Suppression of CD4+ Tconv proliferation in autologous co-cultures

The level of suppression of CD4+ Tconv in the presence of autologous Tregs was compared for all donors and between pairs of twins (Figures 6.10 and 6.11). In contrast to a number of studies (Lindley *et al.*, 2005, Lawson *et al.*, 2008, Brusko *et al.*, 2005, Glisic-Milosavljevic *et al.*, 2007b, Jana *et al.*, 2010), diabetics and controls exhibited similar levels of suppression Unexpectedly, in co-cultures stimulated with 0.25μg/ml anti-CD3 antibody, non-diabetic twins showed significantly higher levels of suppression compared to control donors. Twin pair-wise analysis revealed no differences between index twins and their co-twins in terms of suppression. As naïve Tconv are easier for Tregs to suppress than memory Tconv (Afzali *et al.*, 2011, Jana *et al.*, 2010) the proportion of naïve Tconv in total Tconv populations from each donor was compared to the level of suppression seen, but no correlation was found (Figure 6.12). The level of suppression was also compared to the percentage of monocytes, which showed no

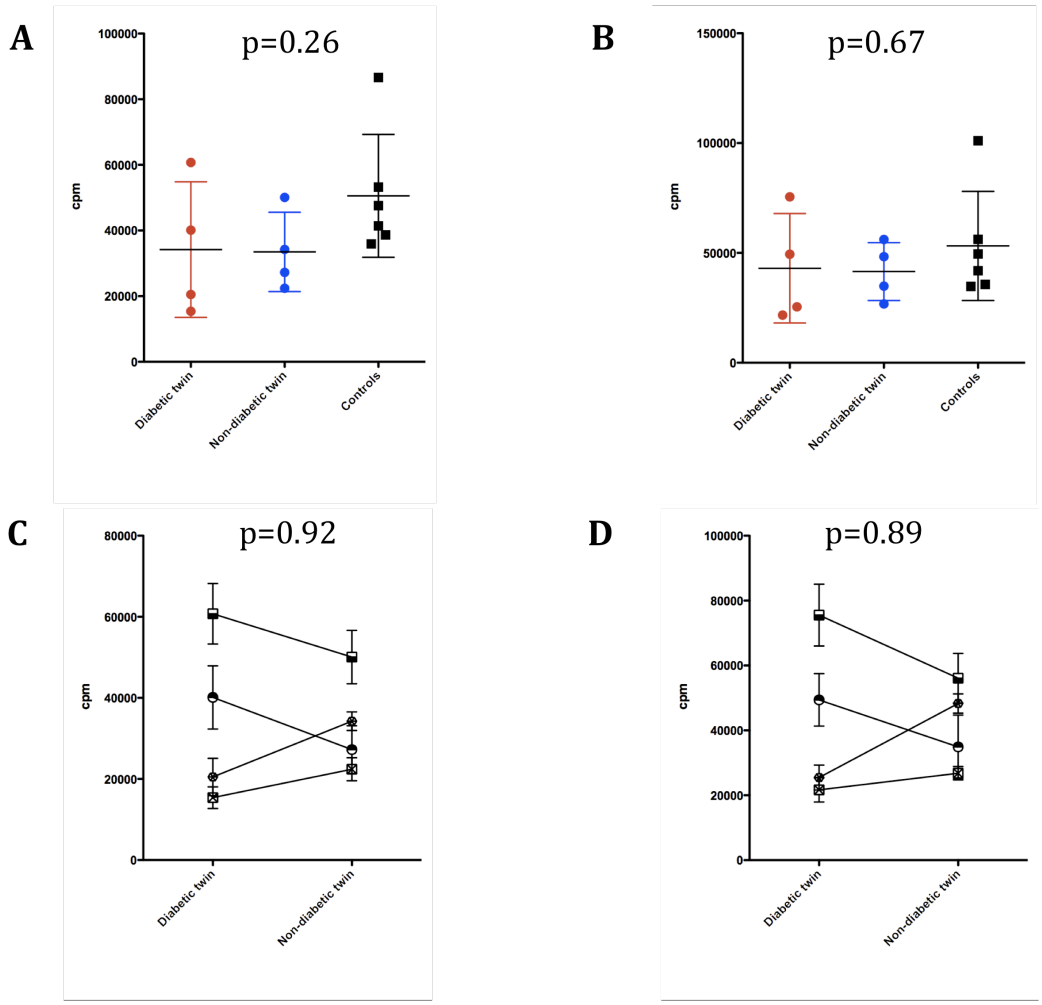

Figure 6.8 Proliferation of CD4+ Tconv when stimulated with plate-bound anti-CD3 antibody *Proliferation of total CD4+ Tconv from all donors is shown in Figures A and B and twin pairs is shown in Figures C and D. Tconv were stimulated with either 0.25µg/ml (Figures A and C) or 0.5µg/ml (Figures B and D) anti-CD3 antibody. Proliferation is shown as mean cpm of 3[H]-thymidine in triplicate wells and error bars show deviation. Red circles represent diabetic twins, blue circles represent non-diabetic twins and the black squares denote control donors. Data in Figures A and B were analysed by a one-way ANOVA whilst the data Figures C and D were analysed by a two-tailed paired t test.*

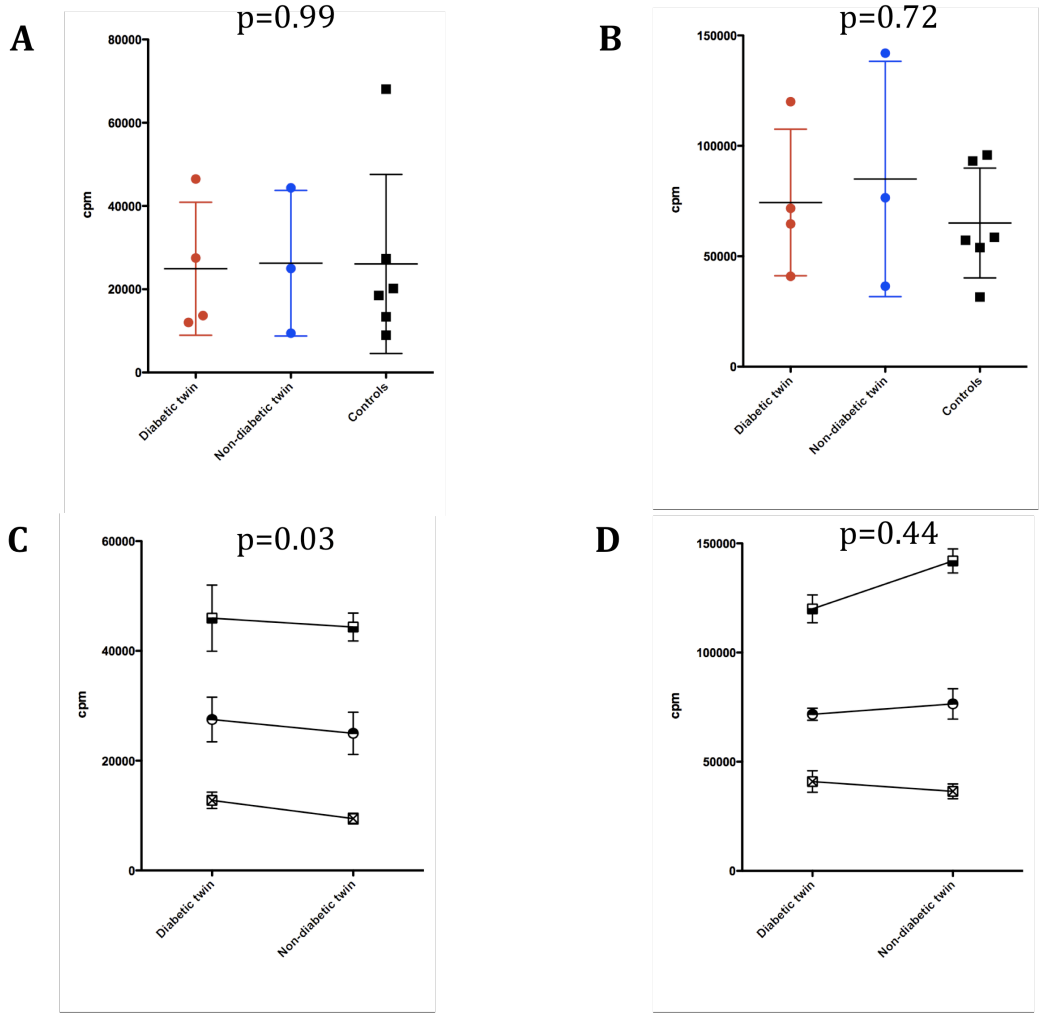

Figure 6.9 Proliferation of CD4+ Tconv when stimulated with Dynabeads® *Proliferation of total CD4+ Tconv from all donors is shown in Figures A and B and twin pairs is shown in Figures C and D. Tconv were stimulated with either Dynabeads® at 1:2.5 (Figures A and C) or 1:1 (Figures B and D). Proliferation is shown as mean cpm of 3[H]-thymidine in triplicate wells and error bars show standard deviation. Red circles represent diabetic twins, blue circles represent non-diabetic twins and the black squares denote control donors. Data in Figures A and B were analysed by a one-way ANOVA whilst the data Figures C and D were analysed by a two-tailed paired t test.*

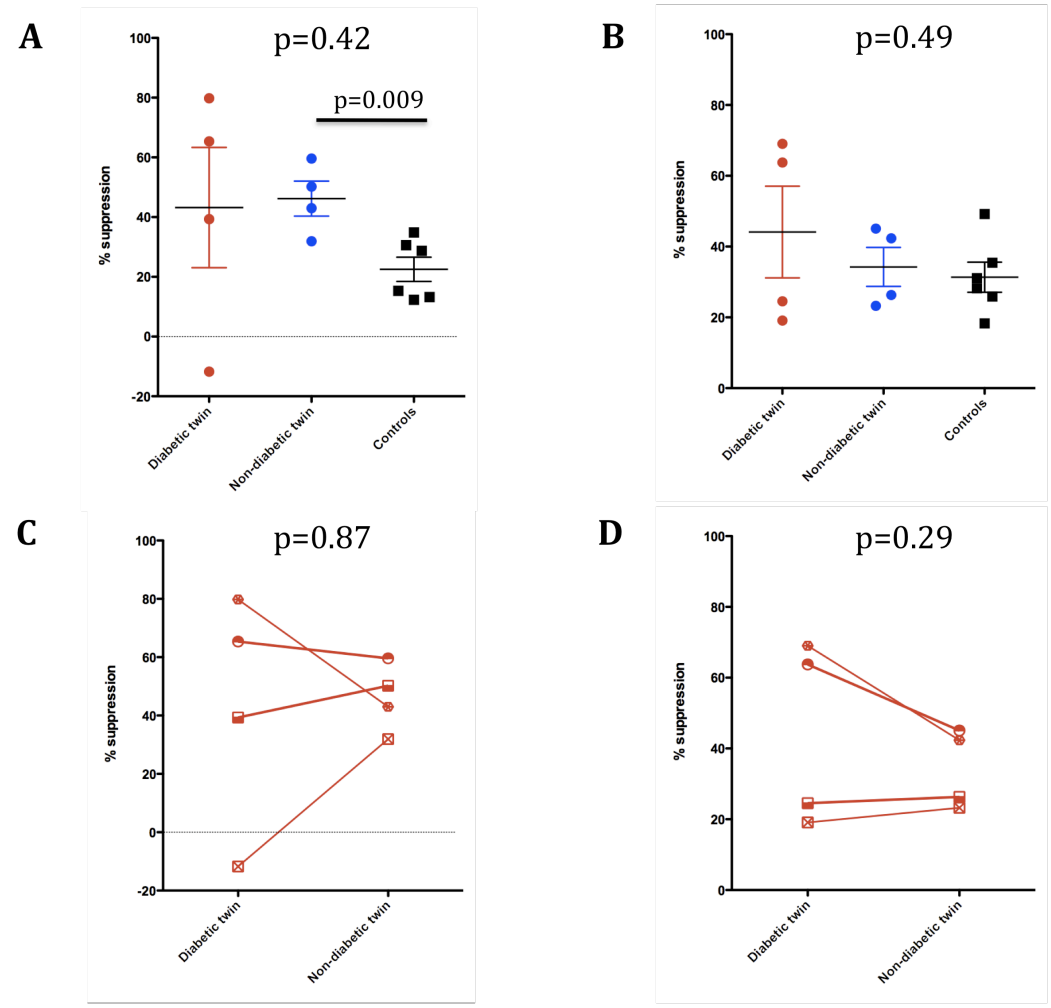

Figure 6.10 Suppression of CD4+ Tconv proliferation in autologous co-cultures stimulated with plate-bound anti-CD3 antibody *The mean level of suppression of total autologous CD4+ Tconv proliferation at a Tconv:Treg ratio of 1:1 from all donors is shown in Figures A and B and from twin pairs is shown in Figures C and D. Suppression was calculated using the formula: % suppression= 100-(counts per minute (cpm) in the presence of Tregs ÷ cpm in the absence of Tregs) x 100). Tconv were stimulated with either 0.25µg/ml (Figures A and C) or 0.5µg/ml (Figures B and D) anti-CD3 antibody. Red circles represent diabetic twins, blue circles represent non-diabetic twins and the black squares denote control donors. Data in Figures A and B were*

analysed by a one-way ANOVA whilst the data Figures C and D were analysed by a two-tailed paired t test. Non-diabetic twins versus controls in Figure A were analysed by a two-tailed unpaired t test.

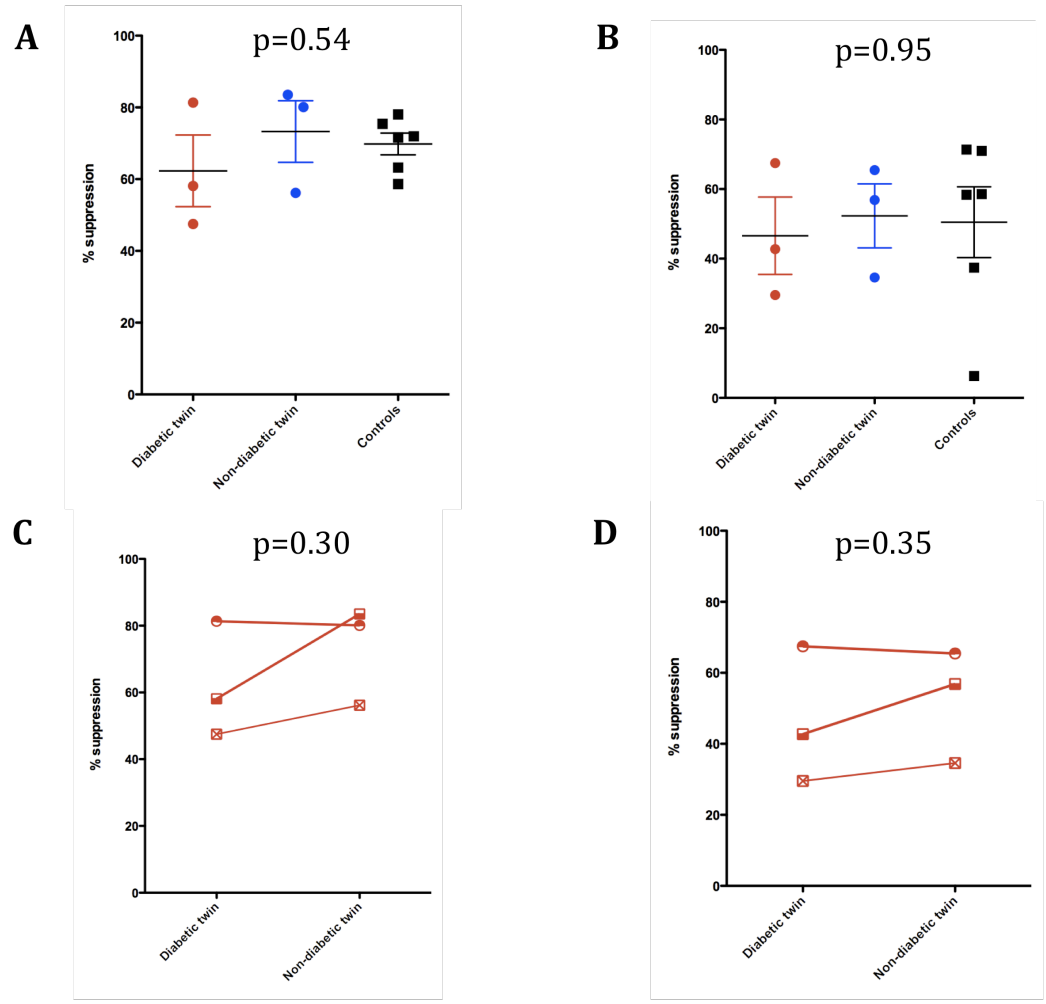

Figure 6.11 Suppression of CD4+ Tconv proliferation in autologous co-cultures stimulated with Dynabeads®

The mean level of suppression of total autologous CD4+ Tconv proliferation at a Tconv:Treg ratio of 1:1 from all donors is shown in Figures A and B and from twin pairs is shown in Figures C and D. Suppression was calculated using the formula: % suppression= 100-(counts per minute (cpm) in the presence of Tregs ÷ cpm in the absence of Tregs) x 100). Tconv were stimulated with either Dynabeads® at 1:2.5 (Figures A and C) and 1:1 (Figures B and D). Red circles represent diabetic twins, blue circles represent non-diabetic twins and the black squares denote control donors. Data in Figures A and B were

analysed by a one-way ANOVA whilst the data Figures C and D were analysed by a two-tailed paired t test.

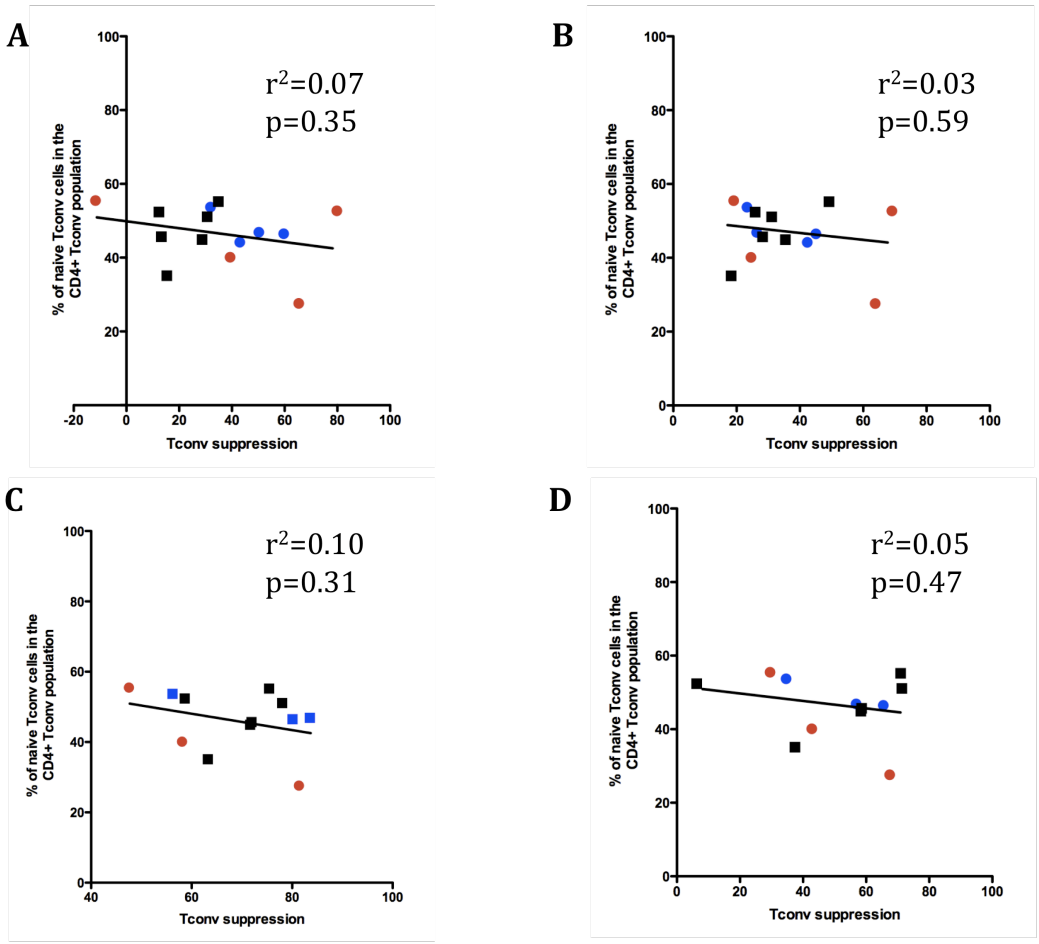

Figure 6.12 Correlation of the percentage of naïve Tconv and the level of suppression

The percentage of naïve CD4+ Tconv was compared to the mean level of Tconv suppression in the presence of Tregs at a Tconv:Treg ratio of 1:1. Co-cultures were stimulated with 0.25µg/ml (Figure A) or 0.5µg/ml (Figure B) anti-CD3 antibody or with Dynabeads® at 1:2.5 (Figure C) and 1:1 (Figure D). Red circles represent diabetic twins, blue circles represent non-diabetic twins and the black squares denote control donors. Correlations were assessed by linear regression with p values showing how significantly non-zero the slope was.

relationship (data not shown).

6.2.8 Suppression of Tconv proliferation in crossover co-cultures

Studies examining defective Treg function in T1D suggest that Tconv from diabetic individuals display increased resistance to Treg suppression (Lawson *et al.*, 2008, Schneider *et al.*, 2008) (Chapter One, Section 1.14). To examine further whether this resistance is genetically-determined, crossover co-cultures were set-up for each twin pair (Figures 6.13 and 6.14). These examined how well Tregs from one twin suppressed Tconv from the other twin, compared to their own. These yielded no significant differences, although under one condition there was a trend for Tregs from non-diabetic twins to control Tconv from diabetic twins better than their own.

6.2.9 Cytokine production by Tconv cultured alone or with Tregs

When stimulated with 0.25μg/ml of anti-CD3 antibody, non-diabetic twins showed significantly higher levels of suppression compared to controls. Therefore, the levels of IFNγ and IL-10 produced by these cultures were also analysed. No differences in either the level of IFNγ production by Tconv in the absence or presence of Tregs were seen (Figure 6.15) nor was there a difference in the suppression of this cytokine (Figure 6.16). The relationship between the percentage of monocytes and both the production

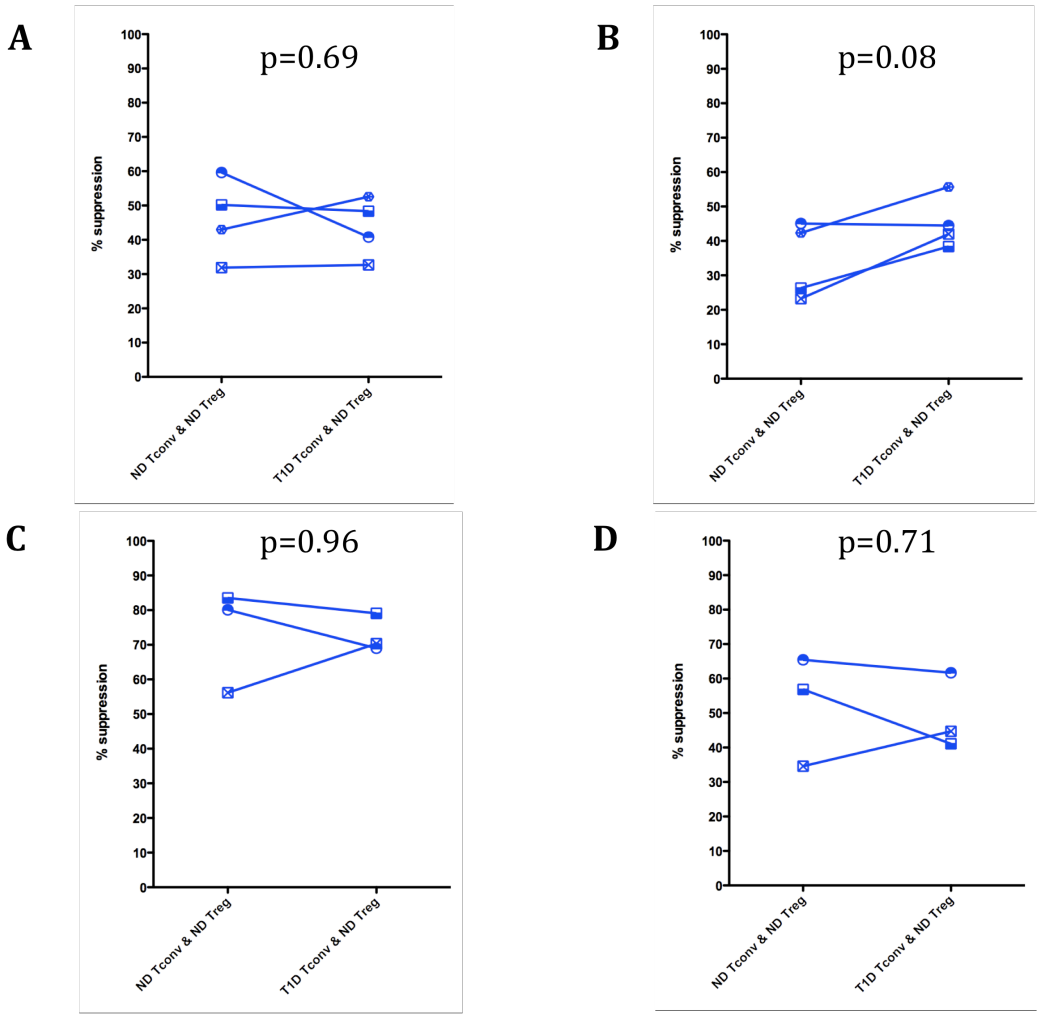

Figure 6.13 Comparison of the ability of Tconv from both diabetic and non-diabetic twins to be suppressed by non-diabetic Tregs

Non-diabetic Tregs were examined for their ability to suppress Tconv from the non-diabetic twin and from the diabetic index twin, at a Tconv:Treg ratio of 1:1. The mean level of suppression is shown and was calculated using the formula: % suppression= 100-(counts per minute (cpm) in the presence of Tregs ÷ cpm in the absence of Tregs) x 100). Co-cultures were stimulated with 0.25µg/ml (Figure A) or 0.5µg/ml (Figure B) anti-CD3 antibody or with Dynabeads® at 1:2.5 (Figure C) and 1:1 (Figure D). Data was analysed using a two-tailed paired t test. ND=non-diabetic twin, T1D=diabetic twin.

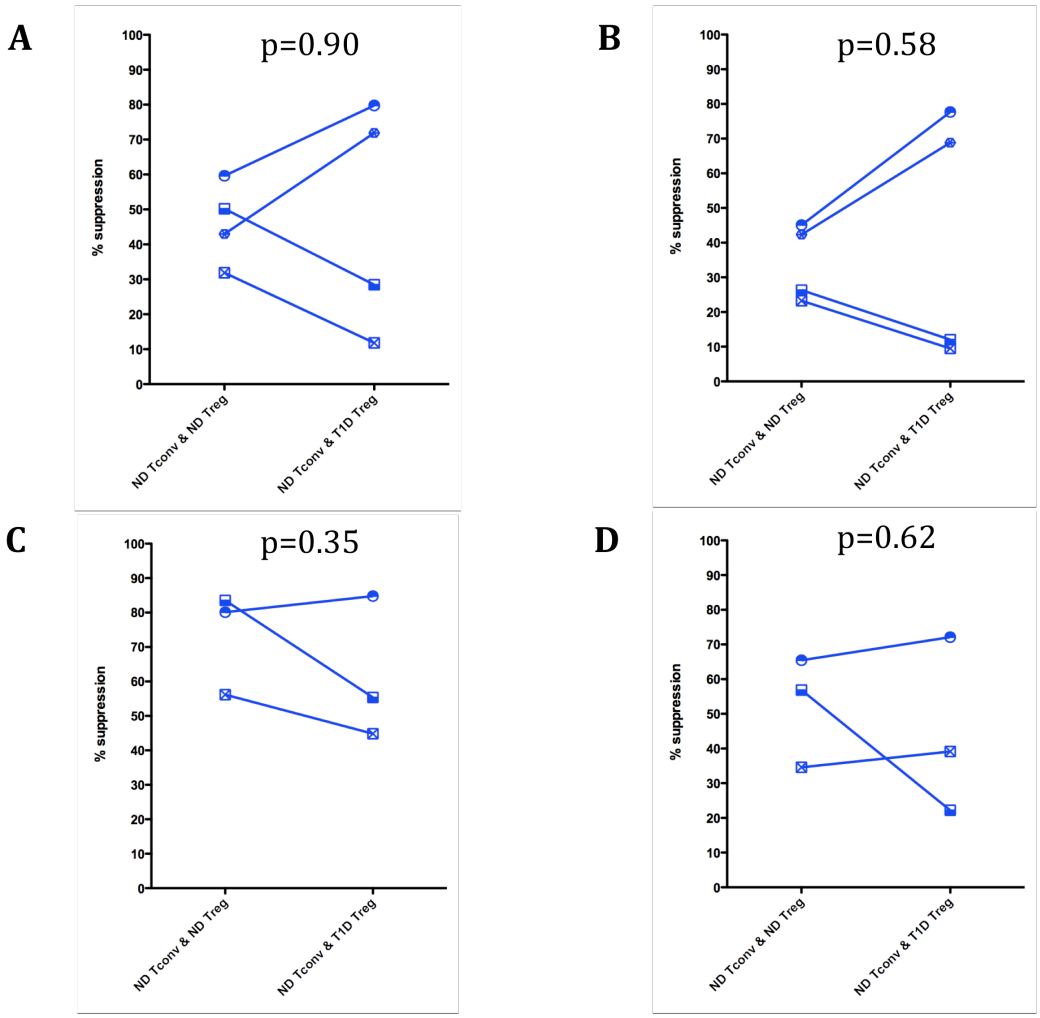

Figure 6.14 Comparison of the ability of Tregs from both diabetic and non-diabetic twins to suppress non-diabetic Tconv *Tregs from both the non-diabetic twin and the diabetic index twin were examined for their ability to suppress Tconv from the non-diabetic twin, at a Tconv:Treg ratio of 1:1. The mean level of suppression is shown and was calculated using the formula: % suppression= 100-(counts per minute (cpm) in the presence of Tregs ÷ cpm in the absence of Tregs) x 100). Co-cultures were stimulated with 0.25μg/ml (Figure A) or 0.5μg/ml (Figure B) anti-CD3 antibody or with Dynabeads® at 1:2.5 (Figure C) and 1:1 (Figure D). Data was analysed using a two-tailed paired t test. ND=non-diabetic twin, T1D=diabetic twin.*

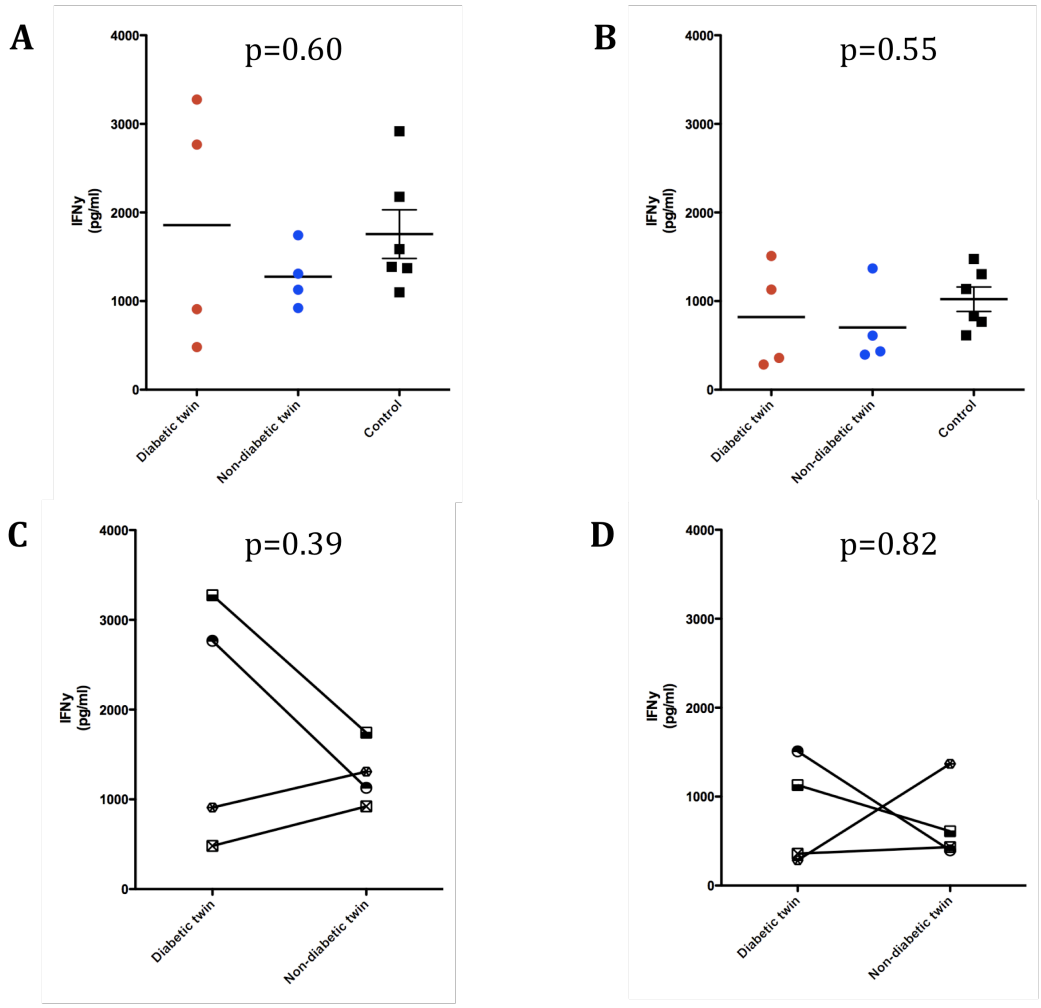

Figure 6.15 IFNγ production in cultures of Tconv either alone or in co-culture with Tregs

The concentration of IFNγ (pg/ml) produced by Tconv cultured alone (Figures A and C) or in co-culture with Tregs (Figures B and D) was measured. The levels are shown for all donors (Figures A and B) and in pair-wise analyses of twins (Figures C and D). All cultures were stimulated with 0.25μg/ml anti-CD3 antibody. Red circles represent diabetic twins, blue circles represent non-diabetic twins and the black squares denote control donors. Data in Figures A and B were analysed by a one-way ANOVA whilst the data Figures C and D were analysed by a two-tailed paired t test.

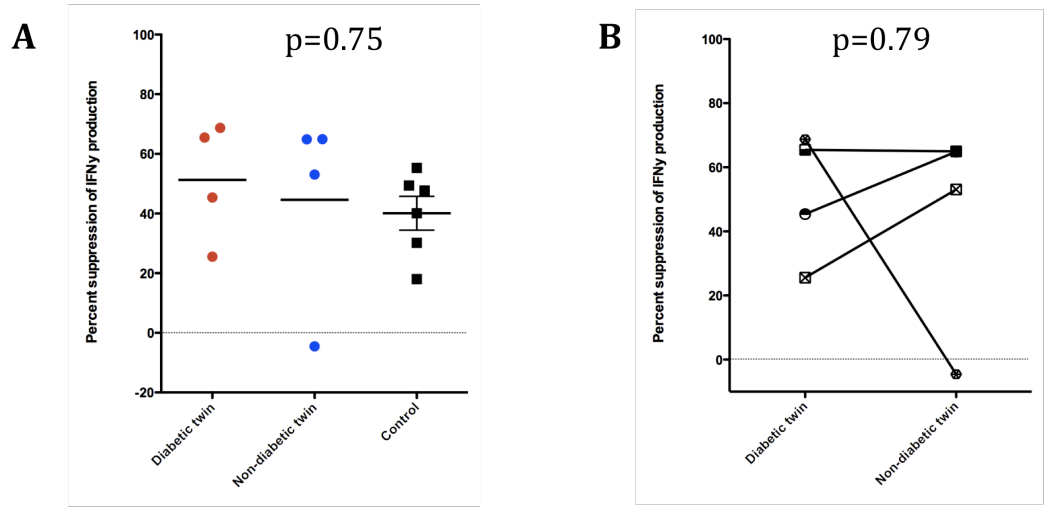

Figure 6.16 Percent suppression of IFNγ production and correlation with percentages of Tregs

The percent suppression of IFNγ production in the presence of Tregs is shown for all donors (Figure A) and for pairs of twins (Figure B) in co-cultures stimulated with 0.25μg/ml anti-CD3 antibody. Red circles represent diabetic twins, blue circles represent non-diabetic twins and the black squares denote control donors. Data in Figure A was analysed by a one-way ANOVA whilst the data Figure B was analysed by a two-tailed paired t test.

and suppression of IFNγ were examined but no correlations were seen (data not shown). The level of IL-10 production by either Tconv alone or in co-culture with Tregs was comparable between all donors (Figure 6.17) and no correlations between naïve Tconv or monocytes were seen in relation to IL-10 production (data not shown).

6.3 Discussion

The purpose of this chapter was to test the hypothesis that non-diabetic co-twins show comparable levels of reduced Treg suppression, as their index twins.

6.3.1 Statistical power

As discussed in Chapter Four Section, 4.1 power calculations determine the minimum number of subjects required to provide adequate statistical power. Therefore prior to commencing this study, power calculations were conducted to determine the minimum number of subjects required and were based on the same information used for the power calculations in both Chapters Four and Five (an earlier study on LST1D undertaken in this laboratory had shown the average level of suppression of Tconv from LST1D was 38% and that from controls was 62%, together with a S.D. of 19%). Therefore the power calculation showed that to be 95% confident of no Type 1 Error (see below) and to be 80% certain of being able to detect a difference, a minimum of ten donors was required (Brant, 2013). As

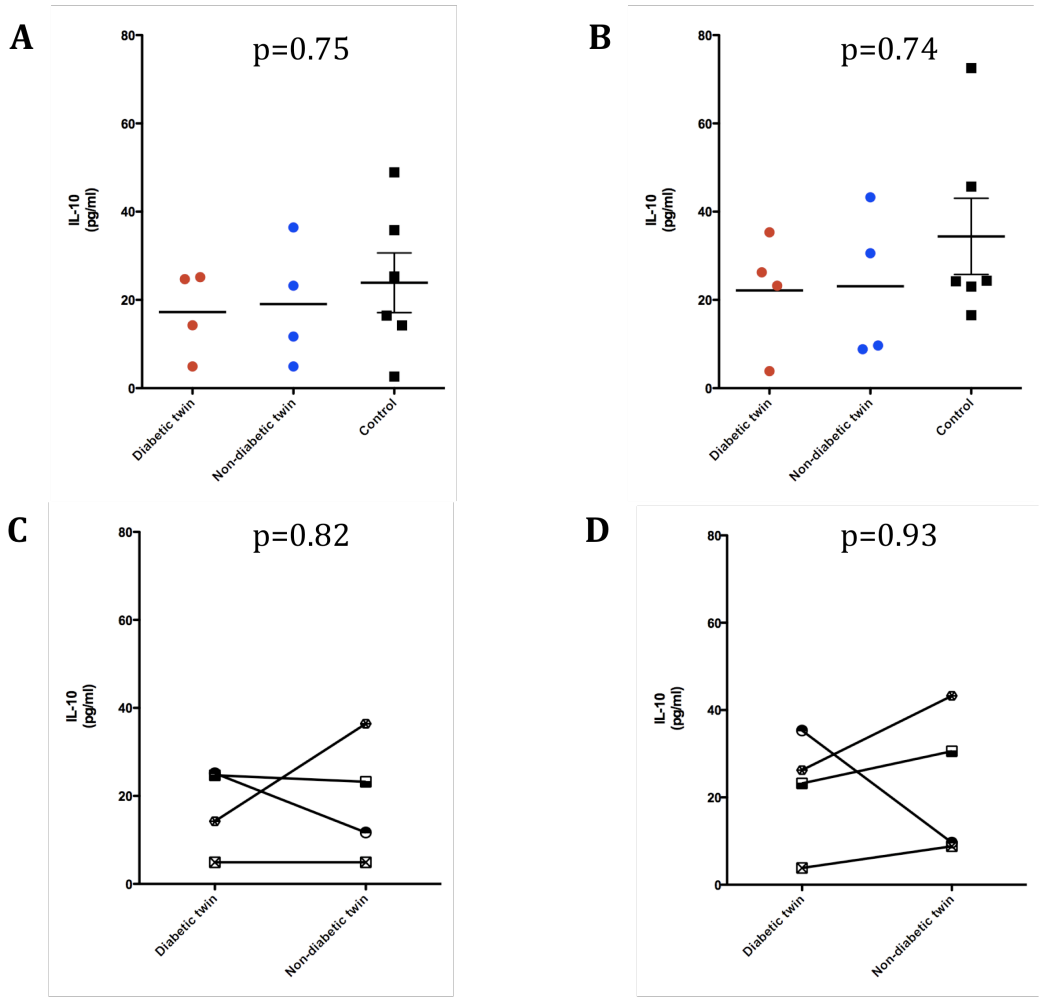

Figure 6.17 IL-10 production in cultures of Tconv either alone or in co-culture with Tregs

The concentration of IL-10 (pg/ml) produced by Tconv in cultured alone (Figures A and C) or in co-culture with Tregs (Figures B and D) was measured. The levels are shown for all donors (Figures A and B) and in pair-wise analyses of twins (Figures C and D). All cultures were stimulated with 0.25μg/ml anti-CD3 antibody. Red circles represent diabetic twins, blue circles represent non-diabetic twins and the black squares denote control donors. Data in Figures A and B were analysed by a one-way ANOVA whilst the data Figures C and D were analysed by a two-tailed paired t test.

explained in Section 6.2.1, only four twin pairs and six controls were assessed. Therefore, the significant differences seen in this study, including: the higher level of Tregs in diabetic twins compared to non-diabetic twins; the higher level of Tconv proliferation when stimulated with Dynabeads® at 1:2.5 in the index twins compared to co-twins and the higher level of suppression in co-twins compared to controls, when stimulated with 0.25μg/ml anti-CD3 antibody, are possibly all Type I Errors. That is the finding of statistical differences that do not actually exist; in other words a false positive (Caramori *et al.*, 2012). In comparison to several other studies, this chapter found no difference in Treg suppression between diabetics and controls (Lindley *et al.*, 2005, Lawson *et al.*, 2008, Brusko *et al.*, 2005, Glisic-Milosavljevic *et al.*, 2007b, Jana *et al.*, 2010). It is possible that due to the low numbers of donors assessed this is a Type II Error, whereby no difference is found, although one does exist (Caramori *et al.*, 2012).

However, another reason why no differences were seen in Treg function may be due to the differences in the procedures in this chapter compared to other studies. As discussed in Chapter Three, compared to earlier studies conducted in this laboratory, the cells in this thesis are isolated by FACS instead of MACS (Lindley *et al.*, 2005, Lawson *et al.*, 2008) resulting in purer populations of Tregs (Tree *et al.*, 2006) (Chapter Three, Section 3.1.1). Although the work by Lawson *et al.* (2008) and Lindley *et al.* (2005) optimised the MACS isolation to select CD25hi cells, memory Tconv, which

display CD25, albeit at lower levels than Tregs (Malek and Bayer, 2004), may still have been selected. Indeed, a study of Treg function in RRMS demonstrated the existence of a defect in patients when the top 4% of CD25 expressing CD4+ T cells were isolated by FACS for suppression assays (Michel *et al.*, 2008). However, this study showed these cells are contaminated with memory Tconv. Therefore, the authors compared their findings with suppression assays using Tregs isolated by gating on the top 2% of CD25 expressing CD4+ T cells, found not to contain Tconv. When this stringent method was used, no difference in suppression could be identified between patients and controls.

Other studies identifying a defect in Treg function in T1D also isolated the Tregs by FACS, by selecting the top 1-2% of CD25 expressing CD4+ T cells (Brusko *et al.*, 2005, Glisic-Milosavljevic *et al.*, 2007b, Jana *et al.*, 2010). However, most of these studies used CD4+ CD25- cells as the responder population (Brusko *et al.*, 2005, Glisic-Milosavljevic *et al.*, 2007b), which largely represents the naïve Tconv population (Malek and Bayer, 2004). Jana *et al* (2010) did compare the suppression of CD25- (naïve) and CD25lo (memory) Tconv, but none of these studies examined the whole Tconv population as in this chapter. Further studies are warranted to examine whether isolating the Tregs by FACS together with suppression assays examining the total CD4+ Tconv population still identify a defect in T1D.

No differences were observed in the percentage of monocytes or B cells

between donors, which corroborated with the findings of other studies comparing diabetics and controls (Habib *et al.*, 2012, Martin *et al.*, 1991). However, in this chapter index twins showed higher proportions of monocytes in relation to B cells, which was close to significance. The relevance of this finding or whether this is a valid result given the low number of twins assessed, remains to be seen. Whilst there are no differences in the number of CD14+ monocytes in T1D, a number of studies have shown differences in the genotype and phenotype of this cell population. Analysis of the gene expression profile of these cells from diabetics show sixteen genes are differentially expressed compared to control donors (Padmos *et al.*, 2008, Beyan *et al.*, 2010). Genes coding for anti-apoptotic proteins are down-regulated, whilst those involved in chemotaxis, adhesion and metabolism are up-regulated. Interestingly, of these sixteen genes, non-diabetic co-twins at very low risk of developing the disease display the same expression levels for thirteen (Beyan *et al.*, 2010).

Also, a defect in the response to a pro-inflammatory signal (lipopolysaccharide (LPS)) has been identified in monocytes from diabetics (at least *in vitro*) (Beyan *et al.*, 2006). In the presence of LPS, these cells were insufficient in their ability to up-regulate cyclooxygenase 2 mRNA, possibly indicating a susceptibility to chronic inflammatory diseases, such as T1D (Beyan *et al.*, 2006). The same defect was seen in the non-diabetic co-twins even though they were unlikely to develop T1D.

Finally, no differences in the production or suppression of IFNγ or the production of IL-10 were found. This was despite a difference between non-diabetic twins and controls in the suppression assay. Again, although more donors are required to confirm this, this finding does fit with the reports of others. Although higher ratios of IFNγ:IL-10 have been reported in ROT1D donors compared to controls (Lindley *et al.*, 2005) no difference has been recorded in LST1D patients (Lawson, 2008), like the diabetic twins examined in this study.

6.3.2 Treg function in T1D-discordant monozygotic twins

As explained above, no differences were found in Treg suppression between diabetics and controls, possibly due to the low number of individuals assessed. Therefore, whilst no differences in Treg suppression are seen between twins, it is not possible to state whether this is an accurate assessment, as analysis of more twin pairs is required before this can be ascertained. Although they share the same genes and early childhood environment, Treg suppression may be different in co-twins compared to diabetics, possibly implicating a role for epigenetics. Indeed differences in the methylation status of 132 genes in CD14+ monocytes have been shown between discordant monozygotic twins (Rakyan *et al.*, 2011). Interestingly, the methylation status of these genes in the index twins matched those of non-twin diabetic donors, both before and at diagnosis, suggesting they pre-date disease onset. The study by Beyan *et al* (2012) shows that with regards

to monocytes, co-twins exhibit gene expression patterns more similar to their index twins than controls. Therefore if the same pattern was found with gene expression in Tregs, it may be possible that co-twins could show levels of Treg suppression that is intermediary between diabetics and controls.

It is also tempting to speculate that non-diabetics could display a higher level of suppression compared to controls, a possible compensatory mechanism, aiding the prevention of diabetes in these twins. However, a higher number of individuals require testing before any difference in suppression can be deemed valid. A number of studies have reported the existence of beta-cell autoimmunity (measured by factors such as insulin sensitivity, the presence of islet autoantibodies *etc.*) in non-diabetic co-twins close to the diagnosis of the index twin (Johnston *et al.*, 1987, Johnston *et al.*, 1989, Beer *et al.*, 1990, Millward *et al.*, 1986, Heaton *et al.*, 1987). These co-twins were prospectively found to be at a very low risk of T1D and in nearly all cases, these signs of beta-cell autoimmunity remitted over time. Perhaps better Treg function in these donors may be implicated in this remission.

6.3.3 Conclusions

Disappointingly, fewer donors than expected were analysed as part of this study. As statistical power could not be reached, it is likely many, if not all, of the significant differences seen in this chapter are Type I or Type II

Errors. Therefore more donors will have to be examined before the hypothesis can be tested.

Chapter Seven: Examination of the IL-2 signalling cascade within pairs of type 1 diabetes-discordant monozygotic twins

7.1 Introduction

7.1.1 Defective IL-2 signalling in T1D

As discussed in detail in Chapter One, Section 1.14.5, IL-2 signalling in T1D has been reported by several studies to be defective (Long *et al.*, 2011, Long *et al.*, 2010, Zier *et al.*, 1984, Roncarolo *et al.*, 1988, Kaye *et al.*, 1986). The work both in Chapter Five and by Buckner and colleagues demonstrates that even in the presence of exogenous IL-2, a defect in the IL-2 signalling cascade in diabetics is still evident *in vitro* (Long *et al.*, 2010, Long *et al.*, 2011). The study in Chapter Five suggested that at least part of the reduced signalling in the presence of IL-2 in memory Tconv and Tregs from donors with the T1D-susceptible *IL-2RA* haplotype may be due to lower levels of CD25 on these cells. Also work by Buckner and colleagues identified a possible defect in signalling via CD122 in the diabetic donors they examined (Long *et al.*, 2010). This defect in IL-2 signalling also involves the significantly reduced production of IL-2 by both NOD mice (Yamanouchi *et al.*, 2007) and diabetic individuals (Zier *et al.*, 1984, Kaye *et al.*, 1986, Roncarolo *et al.*, 1988, Giordano *et al.*, 1989), which appears to be genetically-determined to some extent (Yamanouchi *et al.*, 2007, Kaye *et al.*,

1986).

7.1.2 Tr1 cells

As discussed in Chapter One, Sections 1.2 and 1.5, other populations of regulatory T cells exist besides CD4+ CD25hi FOXP3+ Tregs, such as Tr1 cells (Groux *et al.*, 1997, Kemper *et al.*, 2003). There are several ways in which these cells can be generated *in vitro* from CD4+ Tconv, but their generation by activation of CD3 and CD46 is IL-2-dependent (Kemper *et al.*, 2003, Cardone *et al.*, 2010) thus providing a method for examining the IL-2 signalling cascade in T1D-discordant monozygotic twins.

The study by Cardone *et al.* showed that at low concentrations of IL-2 (less than 5 IU/ml) IFNγ-producing T_H1 cells were generated (Cardone *et al.*, 2010). However, at higher IL-2 concentrations, Tr1 cells were promoted, with decreases seen in the level of IFNγ together with the commencement of IL-10 production. This work suggested the presence of a 'switch' from T_H1 cells to Tr1 cells dependent upon the level of IL-2 present. Interestingly, a defect in this switch has been identified in patients with RA, JA and RRMS (Cardone *et al.*, 2010, Astier *et al.*, 2006) (see Section 7.3.1).

Previous work conducted in this laboratory by Mr. Gabriel Wong, demonstrated that total CD4+ T cells from control donors activated with anti-CD3/46 antibodies (albeit at different concentrations than those used

for the study in this chapter) showed significantly higher percentages of IL-10-secreting cells compared to diabetic donors, when analysed by flow cytometry (Wong, 2008) (Figure 7.1). Therefore the purpose of this chapter was to test the hypothesis that non-diabetic twins would show comparably poor levels of Tr1 cell generation to their index twins. In this chapter, CD4+ Tconv from the donors processed in Chapter Six were used. These cells were first isolated FACS (as described in Chapter Two, Section 2.8.4 and Chapter Three, Section 3.2.1) and activated with anti-CD3/46 antibodies in the presence of several IL-2 concentrations (Chapter Two, Section 2.8.9). Three days later, supernatants were taken and assessed for the presence of IFNγ and IL-10 by ELISAs (Chapter Two, Section 2.8.10). Statistical analyses were performed as described in Chapter Two, Section 2.8.11 and are given in each figure. A summary of all procedures conducted is shown in Figure 7.2.

7.2 Results

7.2.1 Donor selection and isolation of cells

The experiments described in this study were conducted on the same T1D-discordant monozygotic twin and control samples obtained for the study in Chapter Six. As described in Section 6.2.1, a minimum of ten twin pairs and ten control donors were to be examined, however due to unforeseen circumstances, only four pairs of twins and six control donors were actually processed. The control donors were matched according to age, gender and

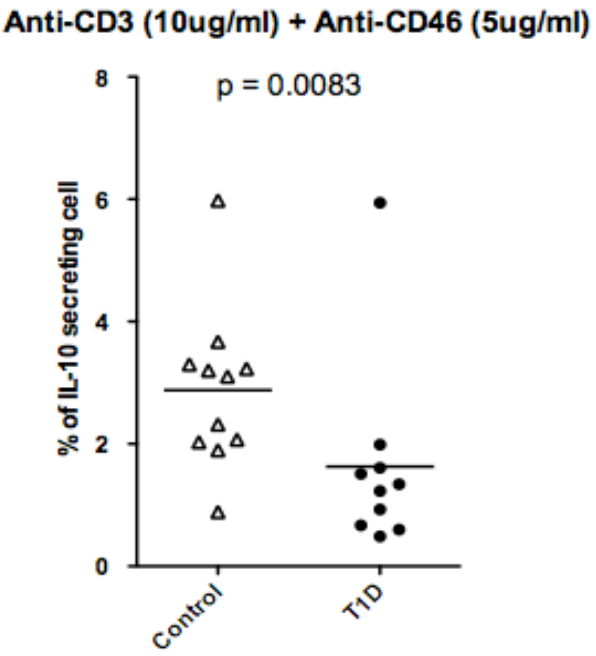

Figure 7.1 Control donors show higher percentages of IL-10-secreting cells compared to diabetics

Following activation of total CD4+ T cells with anti-CD3/46 antibodies, control donors showed significantly higher percentages of IL-10-secreting cells compared to diabetic donors. Data analysed using a two-tailed unpaired t test. Data reproduced from Wong (2008).

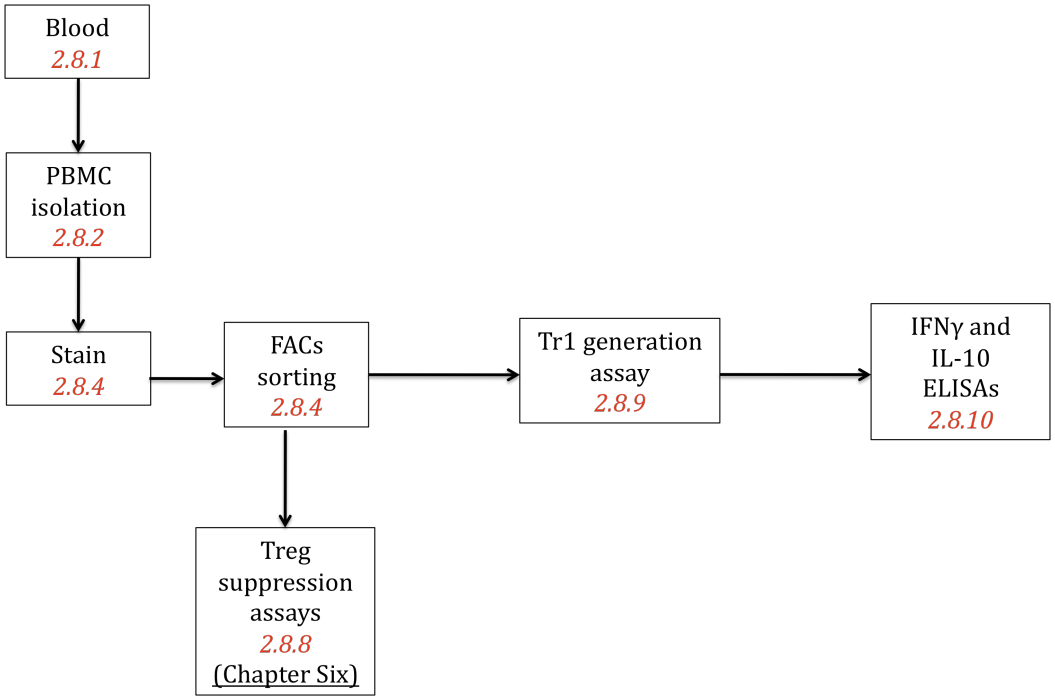

Figure 7.2 Flow chart summarising the procedures conducted on each sample

The above flow chart demonstrates the order and number of all procedures conducted on each pair of samples. The red italics show the section in Chapter Two giving full details of protocols used.

HLA-DRB1 genotype. Tables 7.1 and 7.2 show data on the twin and control donors, respectively. As discussed in Sections 6.1.2 and 6.2.1, the pairs were selected on the basis that the non-diabetic twins were extremely unlikely to ever develop T1D.

The isolation of the cells has been described previously in Section 6.2.2 and the gating strategy is shown in this chapter also (Figure 7.3). The total CD4+ Tconv population was selected according to the high expression of CD127 and negative to intermediate levels of CD25. In addition to examining suppression of CD4+ Tconv proliferation in Chapter Six (Sections 6.2.7 and 6.2.8), the IL-2-dependent generation of Tr1 cells from CD4+ Tconv was assessed in this chapter.

7.2.2 Examination of the CD4+ Tconv population

CD25 is up-regulated within the first few hours of *in vitro* activation (Poulton *et al.*, 1988). Memory CD4+ Tconv express cell surface CD25 (Afzali *et al.*, 2011) and whilst naïve CD4+ Tconv were previously deemed not to express this marker, it has been shown recently that a subpopulation of these cells is CD25+ (Chapter Four) (Pekalski *et al.*, 2013). Also, memory Tconv are activated earlier by treatment with anti-CD3/46 antibodies compared to naïve Tconv (Grossman *et al.*, 2004). Therefore differences in these cell populations could have implications for the Tr1 generation assay. No differences in either the percentages of memory or naïve Tconv or the

Twin pair no.	Symbol	Gender	Age	DRB1 Genotype
1		F	45	DR3 DR4
2		M	25	DR4
3		F	47	DR4 DR6
4		M	57	DR1 DR3

Table 7.1 Details of the monozygotic twin pair donors processed to examine the IL-2 signalling cascade

M=Male, F=Female

Control donor no.	Symbol	Gender	Age	DRB1 Genotype
T001		F	43	DR1 DR4
T030		F	33	DR3
T033		M	37	DR1 DR4
T070		F	44	DR1 DR4
T073		M	31	DR3
T079		M	62	DR4

Table 7.2 Details of the control donors processed to examine the IL-2 signalling cascade

M=Male, F=Female

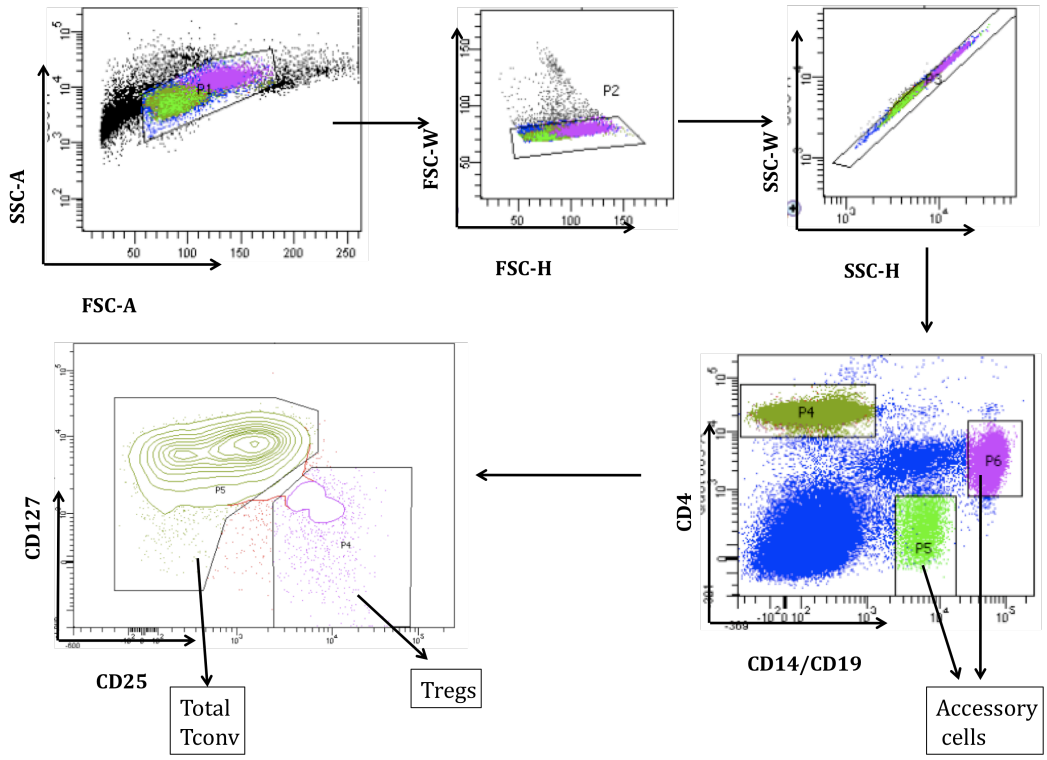

Figure 7.3 Gating strategy for the isolation of Tregs, total CD4+ Tconv, monocytes and B cells

The gating used to isolate cells for the twin study is shown. Firstly, lymphocytes were gated and doublets removed. After gating on CD4+ T cells, the Tconv were isolated by high expression of CD127 and negative to intermediate expression of CD25. Tregs were isolated according to high expression of CD25 correlating with low levels of CD127. Monocytes and B cells were isolated together according to cells that were CD4+ CD14+ and CD4- CD19+, respectively.

ratio of these cells was seen between the sets of donors (as shown in Section 6.2.4).

CD25 forms the high-affinity IL-2 receptor together with CD122 and CD132 (Malek and Bayer, 2004). Therefore the level of expression of CD25 has implications on the IL-2 signalling pathway. No differences in the level of CD25 expression on memory Tconv or the percentage of CD25+ naïve Tconv were seen, although pair-wise analysis of twins revealed a non-significant trend for diabetic twins to possess higher percentages of CD25+ naïve Tconv compared to their co-twins (Figure 7.4). This is reflective of the immunophenotype associated with the susceptible allele at SNP.286 of the IL-2RA gene (Chapter Four). No differences in the level of expression of CD25 on these cells were seen (data not shown).

7.2.3 Generation of Tr1 cells

Tr1 cells generated *in vitro* in the presence of high concentrations of IL-2 are characterised by high levels of IL-10 with equal or higher levels of IFNγ (Cardone *et al.*, 2010). The production of IFNγ (Figure 7.5) and IL-10 (Figure 7.6) from each individual donor was charted. Production of both cytokines increased with increasing IL-2 concentration, which has been reported by others (Cardone *et al.*, 2010). Although in some individuals, production peaked at 20U/ml IL-2, which was noted in several donors tested during the optimisation of this study (Chapter Three, Section 3.2.8).

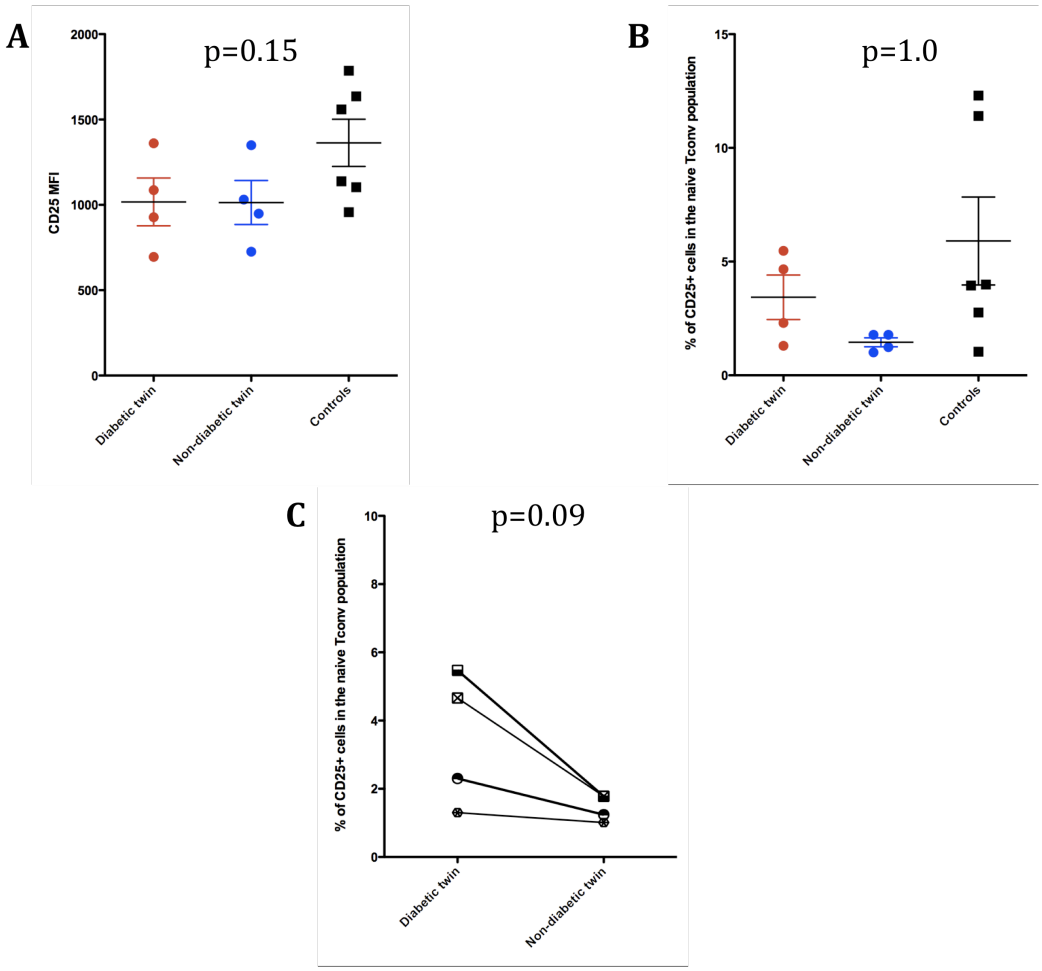

Figure 7.4 Comparison of CD25 expression on CD4+ Tconv

Figure A shows the level of CD25 expression (MFI) on memory CD4+ Tconv and Figure B shows the percentage of CD25+ naïve CD4+ Tconv. Red circles represent diabetic twins, blue circles represent non-diabetic twins and the black squares denote control donors. Figure C shows the pair-wise analysis of CD25+ naïve CD4+ Tconv between twin pairs. Data in Figures A and B was analysed by a one-way ANOVA and data in Figure C was analysed by a two-tailed paired t test.

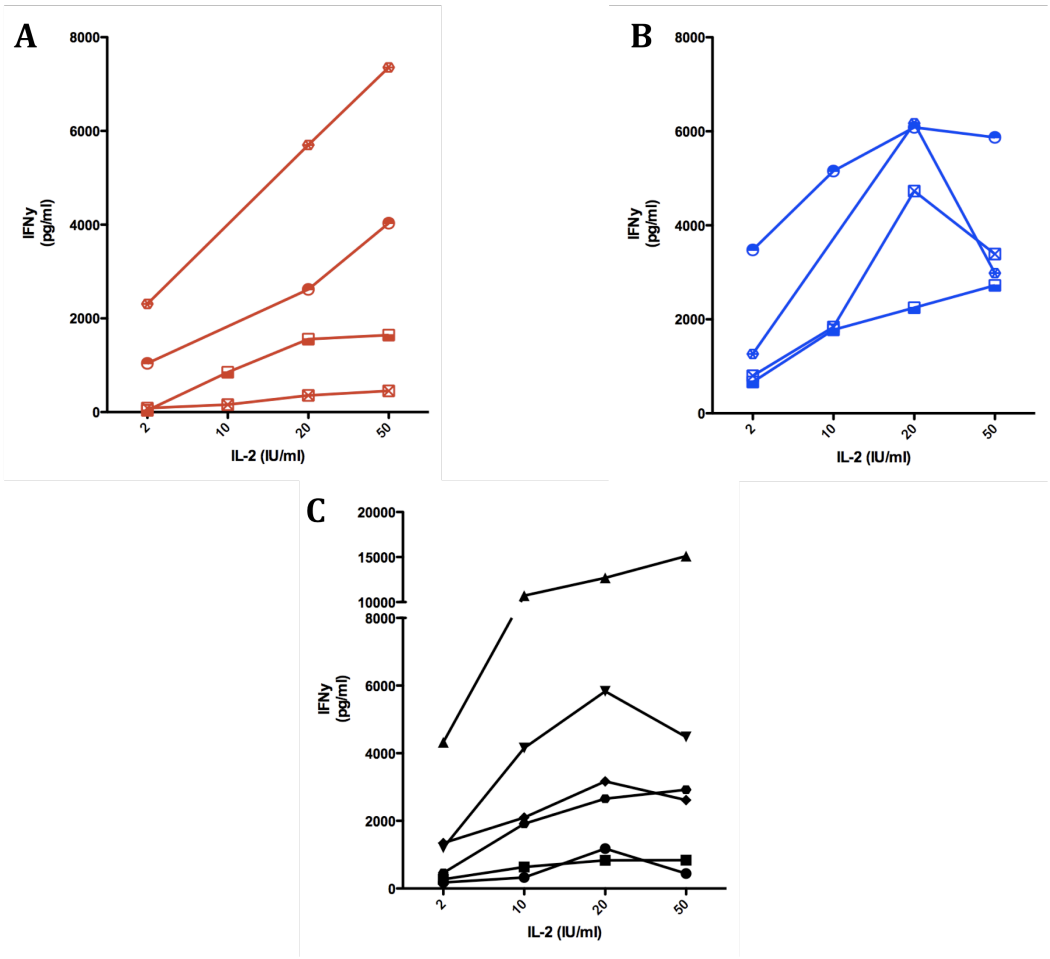

Figure 7.5 Production of IFNγ by Tr1 cells generated from CD4+ Tconv

The level of IFNγ (pg/ml) produced at each IL-2 concentration (IU/ml) by Tr1 cells generated from CD4+ Tconv from each individual is shown. Data is from cells cultured for three days in the presence of anti-CD3/46 antibodies. Figure A shows production by diabetic twins, Figure B shows production by non-diabetic co-twins and Figure C shows production by controls.

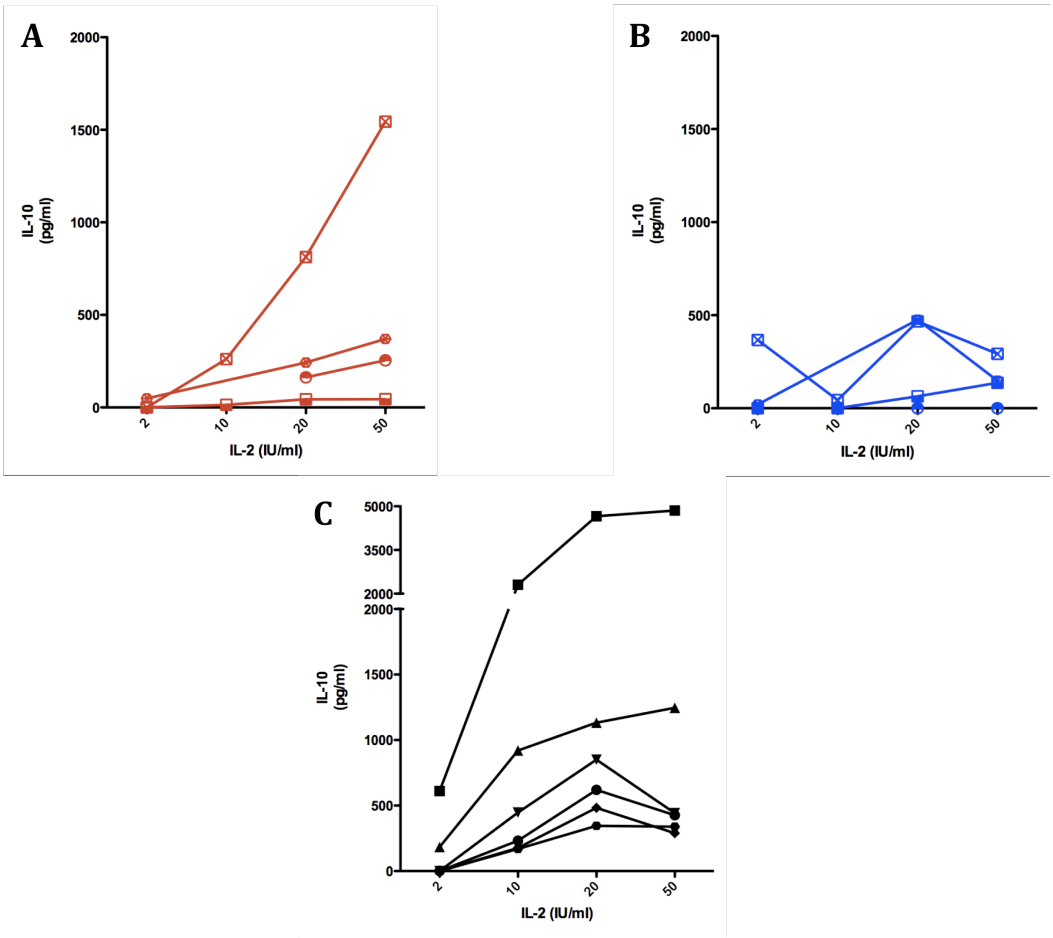

Figure 7.6 Production of IL-10 by Tr1 cells generated from CD4+ Tconv

The level of IL-10 (pg/ml) produced at each IL-2 concentration (IU/ml) by Tr1 cells generated from CD4+ Tconv from each individual is shown. Data is from cells cultured for three days in the presence of anti-CD3/46 antibodies. Figure A shows production by diabetic twins, Figure B shows production by non-diabetic co-twins and Figure C shows production by controls.

A high level of inter-person variation was seen for all donors and analysis of cytokine production revealed no correlation between twins for either the total level of IL-10 or IFNγ produced at any single dose of IL-2 or the maximum cytokine secreted at any dose (Figure 7.7). In some instances the production of IFNγ and IL-10 was highly polarised. For example, the non-diabetic twin from pair one showed very poor responsiveness of IFNγ at all IL-2 concentrations but one of the highest levels of IL-10 production. In comparison, the non-diabetic twin from pair three showed the opposite with high levels of IFNγ but no IL-10 production in response to any IL-2 concentration.

As the majority of control individuals appear to show higher levels of IL-10 production than the twin pairs, the levels of both IFNγ and IL-10 generation from all donors were directly compared. As shown in Figure 7.8, whilst there is no difference in the mean level of IFNγ produced, control donors produced higher levels of IL-10 than diabetic twins (although significance was not reached ($p=0.09$)) and significantly higher levels of IL-10 than non-diabetic twins ($p=0.04$) when Tconv were stimulated in the presence of 50 IU/ml IL-2.

T_H1 cells predominately produce IFNγ, whilst Tr1 cells are characterised by the production of both IFNγ and IL-10 (Cardone *et al.*, 2010). As mentioned above, it has been suggested that activation of Tconv with anti-CD3/46

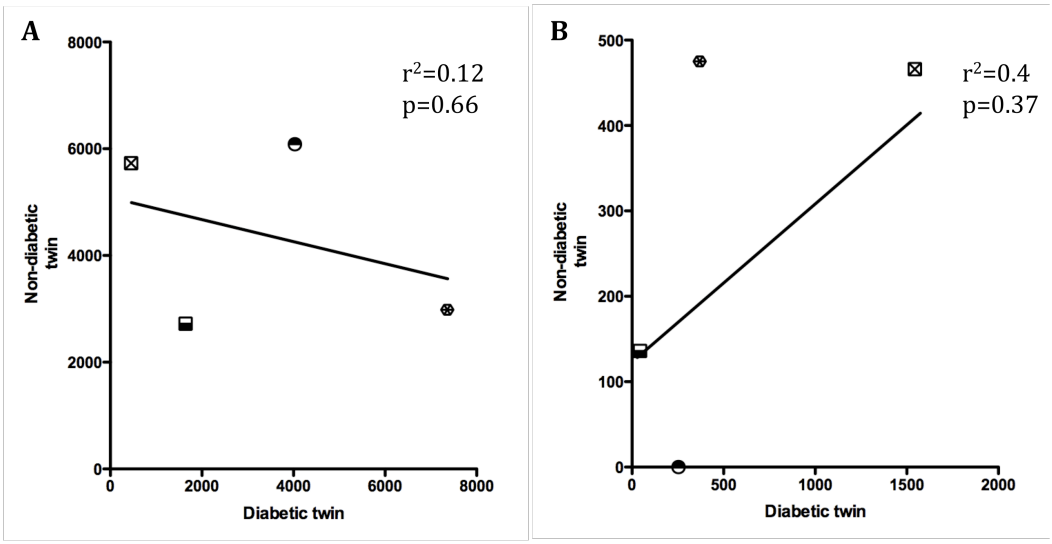

Figure 7.7 Correlations of cytokine production between index and co-twins

The correlation between the highest level of IFNγ (pg/ml) (Figure A) or IL-10 (pg/ml) (Figure B) produced by the index twin and the co-twin is shown. The highest level of IFNγ and IL-10 production (regardless of the IL-2 concentration used) for each twin is plotted. Correlations were assessed by linear regression with p values showing how significantly non-zero the slope was.

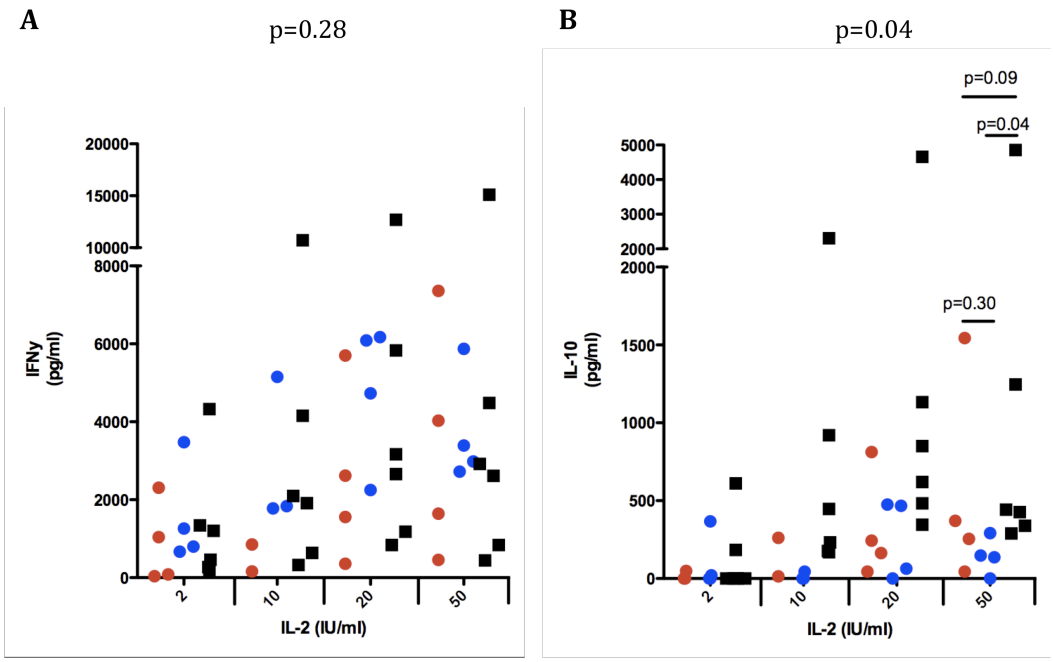

Figure 7.8 Mean level of cytokine production

The mean level of IFNγ production (pg/ml) (Figure A) and IL-10 (Figure B) is shown for the three sets of donors. Data is from cells cultured for three days in the presence of anti-CD3/46 antibodies and 50 IU/ml IL-2. Red circles represent diabetic twins, blue circles represent non-diabetic twins and the black squares denote control donors. Data was measured by a one-way ANOVA. For Figure B at 50 IU/ml of IL-2, data between twins was measured by a two-tailed paired t test and data between control donors versus diabetic or non-diabetic twins was measured by a two-tailed unpaired t test

antibodies in the presence of high IL-2 concentrations results in a T_H1 to Tr1 cell switch, which is reportedly defective in a number of autoimmune diseases (Cardone *et al.*, 2010, Astier *et al.*, 2006). Therefore the ratio of these cytokines was compared for all donors. As shown in Figure 7.9, there were no significant differences in the IFNγ:IL-10 ratios between all three sets of donors and between pairs of twins. There were also no significant correlations between either the memory Tconv CD25 expression or the percentage of CD25+ naïve Tconv with the IL-10 produced in the presence of 50 IU/ml IL-2 (Figure 7.10). Although a non- significant trend between CD25 expression on memory Tconv and IL-10 production was seen.

7.3 Discussion

The IL-2 signalling cascade in T1D is defective (Long *et al.*, 2011, Long *et al.*, 2010) (Chapter One, Section 1.14.5) and possibly some or all of the several T1D-associated genes functioning within this pathway (*i.e. IL-2RA*, *PTPN2*) may be involved (Todd, 2010) (Chapter One, Section 1.13). The generation of Tr1 cells from CD4+ Tconv following activation of CD3 and CD46 in an IL-2-dependent manner provided a method by which the IL-2 signalling cascade could be compared, not only between non-diabetic twins and their diabetic index twins, but also with non-diabetic, non-twin individuals.

7.3.1 Generation of Tr1 cells

Although no differences in the proportion of memory or naïve Tconv were

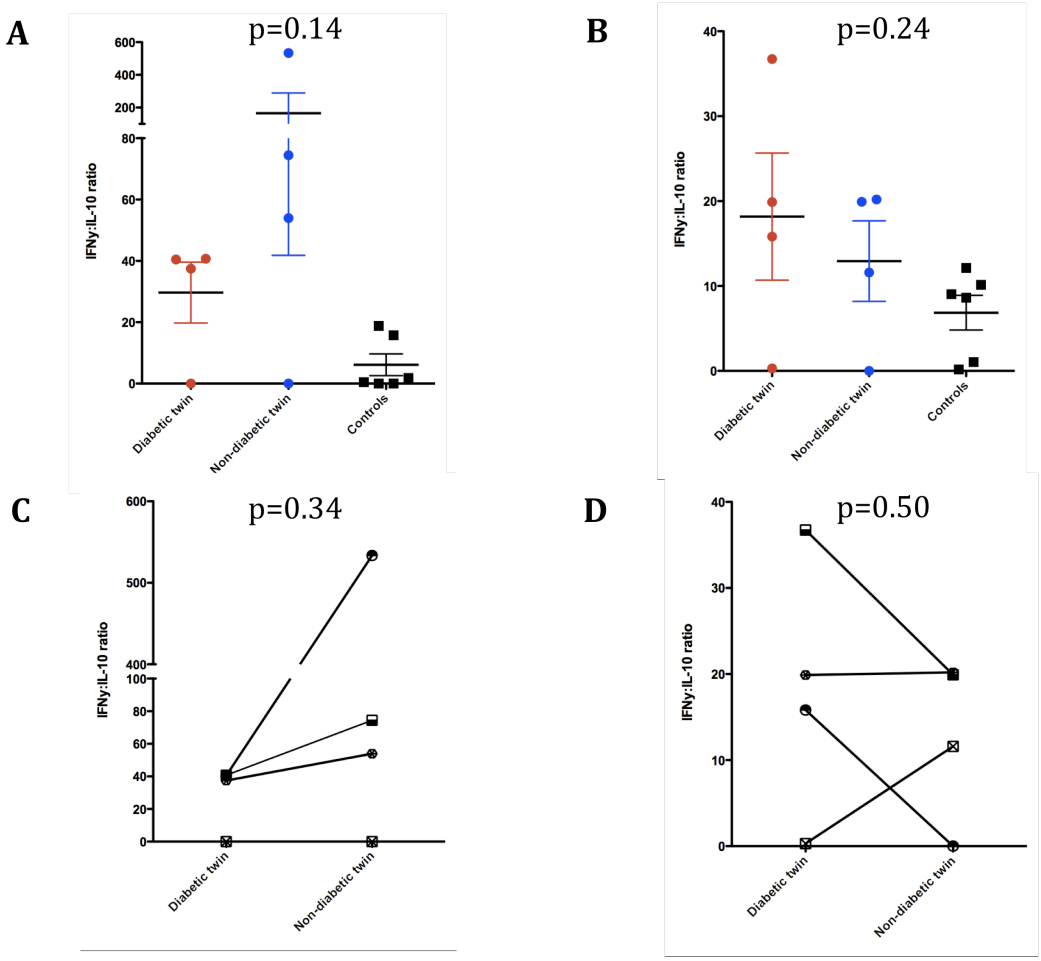

Figure 7.9 IFNγ:IL-10 ratios

The ratio of IFNγ:IL-10 produced by Tr1 cells incubated in 50 IU/ml of IL-2 and stimulated with anti-CD3/28 antibodies (Figures A and C) or anti-CD3/46 antibodies (Figures B and D) for three days is shown. Figures A and B show data from all three sets of donors. Red circles represent diabetic twins, blue circles represent non-diabetic twins and the black squares denote control donors. Figures C and D show twin pair-wise analyses. Data in Figures A and B was analysed by a one-way ANOVA and data in Figures C and D was analysed by a two-tailed paired t test.

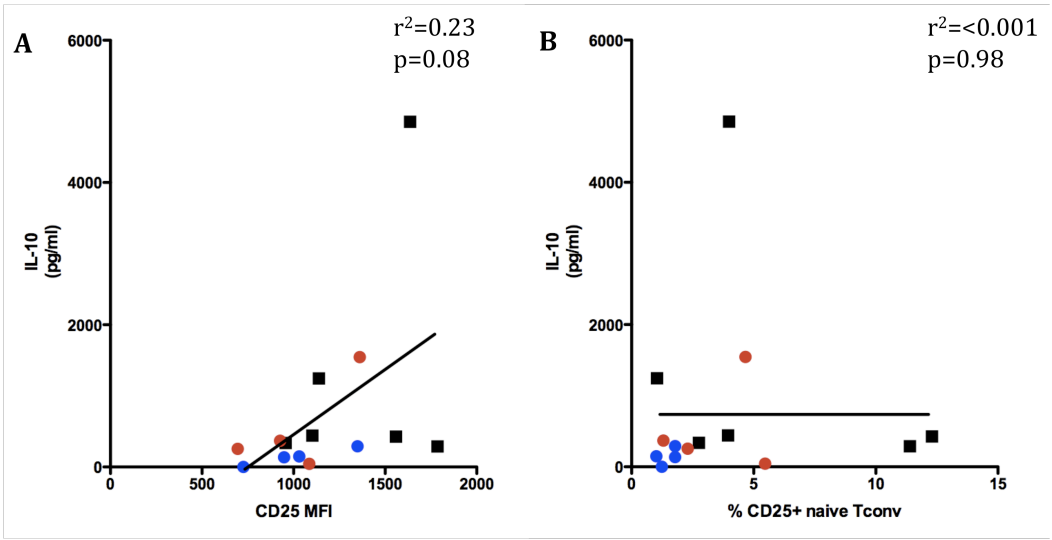

Figure 7.10 Correlation between IL-10 production and CD25 expression

Figure A shows the production of IL-10 (pg/ml) in the presence of 50 IU/ml of IL-2 versus the level of CD25 expression on memory Tconv. Figure B shows the production of IL-10 (pg/ml) in the presence of 50 IU/ml of IL-2 versus the percentage of CD25+ naïve Tconv. Red circles represent diabetic twins, blue circles represent non-diabetic twins and the black squares denote control donors. Correlations were assessed by linear regression with p values showing how significantly non-zero the slope was.

identified between the sets of donors, diabetic twins were found to have higher levels of CD25+ naïve Tconv than their non-diabetic co-twins, although the difference did not reach significance. Interestingly, this is similar to the immunophenotype associated with the susceptible allele at SNP.286 in the IL-2RA gene associated with T1D (Chapter Four, Section 4.1). However, as described in Chapter Six, as so few samples were assessed, statistical power was not reached (Section 6.3.1). Therefore, more twin pairs would need to be assessed to examine if this trend is valid.

The production of both IFNγ and IL-10 from cells stimulated with anti-CD3/46-antibodies was highly variable between donors, including between pairs of twins. There was no clear pattern of cytokine production between twin pairs or to which twin (diabetic or non-diabetic) was the better producer of either IFNγ or IL-10. However, when compared to the control donors, diabetics showed lower levels of IL-10 production, which almost reached significance, whilst non-diabetic twins did display significantly lower levels of IL-10 compared to controls. No significant difference in IL-10 production was seen between twin pairs. Nor were there any significant differences between donor groups in terms of IFNγ production. It would be interesting to determine whether diabetics produce significantly lower levels than controls when more twin pairs are assessed.

A defect in the switch from T_H1 cells to Tr1 cells has been identified in RA, JA

(Cardone *et al.*, 2010) and RRMS patients (Astier *et al.*, 2006). Cardone *et al.* found that RA and JA patients produce much higher levels of IFNγ in relation to IL-10 although in some of these patients, the level of IL-10 was comparable to that produced by control individuals, suggesting that the defect arises from the inability to down-regulate IFNγ (Cardone *et al.*, 2010). However, in RRMS patients, very low levels of IL-10 were produced compared to controls, although no difference in the level of IFNγ was seen (Astier *et al.*, 2006). This suggests there may be more than one defective mechanism resulting in the prevention of the switch to Tr1 cells. Therefore, the defects in the IL-2 signalling cascade in T1D may also cause a defect in the generation of Tr1. However, other factors may be involved, as the defect in Tr1 generation in RRMS patients was linked to an increase in the expression of the CYT-2 isoform of CD46 in these patients compared to controls, which has been shown to be inadequate in inducing IL-10 expression unlike CYT-1 (Cardone *et al.*, 2010) (Chapter One, Section 1.5). Although, to date, no association with polymorphisms in the CD46 gene have been found with T1D (GWAScentral, 2012). The findings in this chapter revealed no differences in the IFNγ:IL-10 ratios across the three donor groups. Therefore it is questionable if the differences seen in IL-10 are suggestive of a defective switch, if comparable proportions of both cytokines are produced by all three groups of donors. Especially as IFNγ may play a role in suppressing T_H17 cells (Apetoh *et al.*, 2010). Again, this study requires more individuals to be analysed before any valid conclusions can

be drawn.

The examination of Tr1 cells has mainly been conducted by generating these cells *in vitro*. This hinders the ability to draw direct conclusions between Tr1 cells and cells from T1D patients, studied *ex vivo*, as there remains the uncertainty that these might not be the same subset of cells. Also, no Tr1 cell-specific marker has yet been identified. Analysis of CD4+ T cells recognising peptides from IA-2 and pro-insulin (PI) revealed that the majority of these cells from non-diabetics produced high levels of IL-10 with little or no IFNγ with the opposite cytokine expression profile seen in diabetics (Arif *et al.*, 2004). Interestingly, the minority of diabetics whose cells produced high levels of IL-10 were significantly older at diagnosis compared to those whose cells secreted mainly IFNγ. Although the phenotype of these cells was not studied in this report, these are characteristics of Tr1 cells and the production of a pro-inflammatory response may be due to defective IL-2 signalling.

Later work whereby these cells, termed IL-10–secreting islet-specific (ISIS) Tregs were isolated and cloned from non-diabetic individuals, revealed that they are Tr1-like in terms of high levels of IL-10 secretion and suppression in a perforin- and granzyme B-dependent manner (Tree *et al.*, 2010). However, unlike previous reports of Tr1 cells, these cells also produced granzyme A and, at least in this study, suppressed in an IL-10-independent

manner. Also ISIS Tregs are FOXP3 positive, which is a marker reportedly absent in Tr1 cells (Apetoh *et al.*, 2010). Therefore it remains unclear as to which cell population ISIS Tregs belong to or if they are a novel subset.

Using the same panel of IA-2 and PI peptides as Arif *et al.* (2004) another study revealed that GADA and IA-2-autoantibody-positive FDR of ROT1D patients exhibited a pro-inflammatory prolife, as measured by IFNγ production, which was higher than control individuals but lower than that of T1D donors (Petrich de Marquesini *et al.*, 2010). However, IL-10 production showed the opposite with higher levels being produced than diabetic individuals but lower than controls. Also, the IFNγ:IL-10 ratios were at an intermediate level between that of controls and diabetics (Petrich de Marquesini *et al.*, 2010). This suggests the production of both cytokines may, to some degree, be genetically determined, possibly involving IL-2 production, although no studies to date have examined IL-2 signalling between diabetics and non-twin, FDR.

7.3.2 Conclusions

Although analysis of more twin pairs is required, the data suggest both diabetic and non-diabetic twins produce lower levels of IL-10 compared to control donors. The relevance of this is not yet clear. Although others have shown these cells can suppress via an IL-10-dependent mechanism, twin donors show comparable IFNγ:IL-10 ratios as controls, suggesting this

decrease in IL-10 production may not reflect a defective switch from T_H1 cells to Tr1 cells. However the lower production of IL-10 in both diabetic and non-diabetic twins, compared to controls, suggests a defect(s) in the IL-2 signalling cascade may be present in the non-diabetic twin also.

Chapter Eight: General discussion

8.1 Summary of thesis findings

The work presented in this thesis attempted to elucidate whether defective Treg function in T1D is primarily a causative factor of this disease or a consequence of this condition. The genotype-immunophenotype study examining the Protective *IL-2RA* P1 haplotype did indeed demonstrate that non-diabetic individuals homozygous for this haplotype had significantly increased Treg fitness and function compared to non-diabetic susceptible homozygotes (Chapter Five). Unfortunately the study of Treg function in T1D-discordant monozygotic twins did not yield any significant information as to whether this defect is genetically-determined as too few twin pairs were analysed (Chapter Six). Finally, the IL-2 signalling cascade was compared in T1D-discordant monozygotic twins by means of an IL-2-dependent Tr1 cell generation assay (Chapter Seven), which suggested the presence of a diminished T_H1-Tr1 switch in both diabetic and non-diabetic twins compared to individuals with no family history of T1D. Reduced IL-2 signalling has implications not only for Tr1 cells, but also for CD4+ CD25hi FOXP3+ Tregs. A better understanding of the interplay between genetic predisposition and environmental factors in T1D (detailed in Chapter One, Section 1.13) and the exact cause of defective Treg function will enable the production of future therapies.

8.2 Possibilities for therapeutic interventions in T1D

8.2.1 Low-dose IL-2 therapy

In an attempt to correct the defect in IL-2 production and signalling in T1D (Zier *et al*., 1984, Kaye *et al*., 1986, Roncarolo *et al*., 1988, Long *et al*., 2010, Long *et al*., 2011), studies in the NOD mouse demonstrated low-dose IL-2 therapy could prevent and even reverse T1D in NOD mice (Grinberg-Bleyer *et al.*, 2010, Tang *et al.*, 2008); an effect attributed to better maintenance of Tregs. However, a ten-fold higher dose exacerbated beta-cell destruction and lowered the survival of these animals (Tang *et al.*, 2008), most likely due to the simultaneous increase in CD4+ and CD8+ Tconv and NK cells alongside the increase in Tregs, thus demonstrating the importance of the IL-2 concentration used. Early-phase clinical trials examining responses to low-dose IL-2 therapy in patients with graft-versus-host disease (GvHD) (Koreth *et al*., 2011, Matsuoka *et al*., 2013) and hepatitis C virus-induced vasculitis (Saadoun *et al.*, 2011) have been conducted, both showing a significant increase in the number of Tregs and Treg:Tconv ratios throughout the course of the study and weeks after the therapy ceased. In the majority of the patients, the symptoms either disappeared or were reduced in their severity.

Following *in vivo* studies in the NOD mouse (Rabinovitch *et al.*, 2002), rapamycin and low-dose IL-2 combination therapy was administered to ROT1D patients, which lead to an increase in the frequency of Tregs and a

prolonged improvement to the IL-2 response by CD25+ cells (Long *et al.*, 2012). However, a transient, yet significant reduction in beta-cell function (as measured by fasting insulin-connecting peptide (C-peptide) levels) was seen in patients throughout the period the therapy was administered. This loss of beta-cell function was not in seen NOD mice (Rabinovitch *et al.*, 2002), demonstrating the caution required when translating treatment from animal models to humans (Chapter One, Section 1.12.1). This adverse effect is thought to be due to administration of IL-2 and not rapamycin, as it was witnessed in participants who did not undergo the full course of rapamycin and rapamycin monotherapy has previously been shown to improve beta-cell function in LST1D (Piemonti *et al.*, 2011). However, the exact cause of this beta-cell loss remains unknown.

It is possible further research can overcome the adverse effects seen in the T1D clinical trial (for example it may be due to the higher daily doses of IL-2 given in this trial compared to the GvHD and HCV-induced vasculitis studies), although important questions remain regarding the suitability of low-dose IL-2 therapy for T1D. Whilst GvHD (Koreth *et al.*, 2011) and HCV-induced vasculitis (Saadoun *et al.*, 2011) are characterised by significantly lower numbers of Tregs, T1D is not (Liu et al., 2006b, Lindley et al., 2005, Lawson et al., 2008, Brusko et al., 2005, Putnam et al., 2005, Ferraro et al., 2011). Also, the defect in Treg function (Lindley et al., 2005, Lawson et al., 2008, Brusko et al., 2005, Jana et al., 2010, Glisic-Milosavljevic et al., 2007b) can be attributed, at least in part, to diminished IL-2 signalling (Long *et al.*,

2010, Long *et al.*, 2011). The work conducted in this thesis demonstrated that at low-doses of exogenous IL-2 but not at higher, possibly saturating doses, individuals with the Protective P1 haplotype showed higher levels of IL-2 signalling (in terms of pSTAT5a) compared to those with the Fully Susceptible haplotype. It is not clear how these concentrations extrapolate to the doses used in the low-dose IL-2 therapy clinical trails conducted so far and whilst the T1D clinical trial demonstrated IL-2 responsiveness is significantly increased in patients with previously reduced signalling, the possibility remains that lower doses of IL-2 will not be able to remedy the diminished IL-2 signalling cascade seen in this disease (Long *et al.*, 2012).

Not all T1D patients exhibit a diminished IL-2 signalling cascade (Roncarolo *et al.*, 1988, Long *et al.*, 2010), raising the suggestion of whether individuals should be assessed for this factor before being given low-dose IL-2 therapy. These patients may be more responsive to this treatment; although this is presently unknown as such individuals were not assessed in the recent T1D clinical trial (Long *et al.*, 2012). Alternatively, it may be the case that T1D patients with sufficient IL-2 signalling may also fall into the category of diabetics who do not exhibit a defect in Treg function (Lindley et al., 2005, Lawson et al., 2008, Brusko et al., 2005, Jana et al., 2010, Glisic-Milosavljevic et al., 2007b) possibly making such therapy superfluous.

8.2.2 Adoptive transfer of *ex vivo* expanded Tregs

Another possibility, which is currently undergoing optimisation by several researchers in the field, is the expansion of *ex vivo* Tregs with a view to adoptively transfer these cells into patients. Numerous studies have shown that Tregs can be expanded *in vitro* (as they were in this thesis, Chapter Two, Section 2.8.7) and in fact suppress to a higher degree than fresh Tregs (Tang *et al.*, 2004, Nishimura *et al.*, 2004, Golovina *et al.*, 2011). Studies in mice have demonstrated *ex vivo* expanded Treg are capable of preventing T1D in prediabetic NOD mice and can even reverse T1D in newly-diabetic mice (Tang *et al.*, 2004). *In vitro* expansion of humans Tregs from both donors with and without T1D has been demonstrated to be possible (Battaglia *et al.*, 2006, Golovina *et al.*, 2011, Putnam *et al.*, 2009).

Promising results have been obtained by Trzonkowski and colleagues when attempting to translate these methods into man. In a study of a patient with GvHD a single infusion of *ex vivo* expanded Tregs from the graft donor showed an improvement in clinical outcomes, reducing the severity of GvHD symptoms (Trzonkowski *et al.*, 2009). Further research by this group demonstrated that autologous *ex vivo* expanded Tregs improved beta-cell function in ROT1D when adoptively transferred within two months of diagnosis (Marek-Trzonkowska *et al.*, 2012). Four months later, the level of C-peptide was significantly higher compared to ROT1D who did not receive this treatment and the use of exogenous insulin significantly lower (not being required at all in two out of ten subjects).

It should be noted though that polyclonal Tregs were used in both of these studies, which have been shown to have less efficacy than antigen-specific Tregs (Sagoo *et al.*, 2011, Tang *et al.*, 2004). The low number of antigen-specific Tregs isolated from a donor, prior to expansion is a limiting factor. One solution is the use of 'Designer Tregs' whereby antigen-specificity can be altered by lentiviral TCR gene transfer into a population of polyclonal nTregs, which have been expanded *in vitro* (Brusko *et al.*, 2010). This also has the benefit of eliminating any T1D-associated polymorphisms present in donor Tregs (Thompson *et al.*, 2012).

There are still concerns with this therapy, such as the plasticity of Tregs. Tregs secreting IFNγ and IL-17 (Miyara *et al.*, 2009, Scotta *et al.*, 2012, Fletcher *et al.*, 2009, Duhen *et al.*, 2012) have been identified in humans and it is unclear whether *ex vivo* expanded Tregs could also begin to produce these cytokines after being administered to patients. Furthermore, whilst it has been shown that Tregs expanded *ex vivo* from T1D patients no longer show a defect in function (Putnam *et al.*, 2009), it is possible that this defect could re-occur post-administration due to defective IL-2 signalling. Again, perhaps patients should be screened for any defects in IL-2 signalling and Treg function prior to treatment. Also, there may be scope for a possible 'combi-therapy' whereby both expanded Tregs and low-dose IL-2 therapy are administered.

8.2.3 Antigen-specific immunotherapy

Administration of antigen-specific peptides recognised by islet autoreactive Tconv have been successful in mouse models of T1D. Oral administration of insulin was shown to reduce insulitis and disease onset in NOD mice (Zhang *et al.*, 1991) and a model of virus-induced T1D (von Herrath *et al.*, 1996) and an altered peptide ligand of the insulin B chain epitope ($B_{9\text{-}23}$) (NBI-6024) (containing two alanine substitutions at residues 16 and 19) delayed onset and reduced the incidence of T1D occurrence (Alleva *et al.*, 2002). Similar results were also found when GAD65 was administered to prediabetic mice (Tisch *et al.*, 1998).

The majority of these studies suggest the therapies are due to the inducement of a T_H2 response (Alleva *et al.*, 2002, von Herrath *et al.*, 1996, Tisch *et al.*, 1998), however the possible role of CD4+ CD25+ FoxP3+ Tregs was not examined. Although the exact mechanism involved in inducing tolerance following peptide administration remains to be elucidated, it is possible it could involve induction of anergy of autoreactive T cells or their deletion and/or Tr1 cell generation (Peakman and Dayan, 2001). In support of this role of Tr1 cells is a study following a group of beekeepers over the course of several beekeeping seasons (Meiler *et al.*, 2008). At the start of each season, the initial exposure to bee venom results in the proliferation of antigen-specific T_H1 and T_H2 cells, but following numerous bee stings throughout the season, these cells switch to IL-10-producing Tr1 cells. As demonstrated in this thesis as well as reports by others, IL-2 is necessary for

the generation of these cells (Kemper *et al.*, 2003, Cardone *et al.*, 2010). Once more, it needs to be questioned as to whether T1D patients should be screened for a defect in IL-2 prior to this therapy being given or whether IL-2 should be administered alongside these peptides.

The translation of these studies into humans has not been quite so promising. All therapies that have so far undergone clinical trial have been generally well tolerated with no associated toxicity or serious adverse effects (Wherrett *et al.*, 2011, Walter *et al.*, 2009, Thrower *et al.*, 2009, Skyler *et al.*, 2005, Alleva *et al.*, 2006, Orban *et al.*, 2010, Ludvigsson *et al.*, 2008) although with generally little effect on clinical outcome and none report a decrease in the amount of exogenous insulin required. A summary of the clinical trials conducted so far is shown in Table 8.1.

The consistent finding that these treatments are well tolerated retains hope for this method of treatment. More studies are required to determine the optimal dose and route of administration as well as the desired frequency of administration (Peakman and von Herrath, 2010). One of the debates in peptide immunotherapy is when treatment should commence. Prior to T1D onset, a higher number of beta-cells can be saved however there are concerns this may exacerbate beta-cell autoimmunity (Pozzilli and Strollo, 2012). A study by Thrower *et al.* (2009) demonstrated that a cohort of LST1D subjects did not exhibit any pro-inflammatory response to PI, however, such responses can be seen in ROT1D donors (Arif *et al.*, 2004).

Peptide	Subject group	Safety & Toxicity	Effect on Beta-cell function	Other outcomes	Reference
Oral insulin	High-risk relatives	Well tolerated. No associated toxicity or serious adverse effects.	None	Slower disease onset rate in subjects with high insulin antibody titres, whilst therapy sustained.	(Skyler *et al.*, 2005) Follow-up study: (Vehik *et al.*, 2011)
NBI-6024	ROT1D	As above.	None.	Shift from a T_H1 to a T_H2 cytokine profile	(Alleva *et al.*, 2006)
NBI-6024	ROT1D	As above.	None.	None.	(Walter *et al.*, 2009)
Insulin B-chain	ROT1D	As above.	None.	Increase in insulin-specific Tregs.	(Orban *et al.*, 2010)
PI peptide	LST1D	As above.	Glycaemic control improved in low-dose peptide group. No decrease in exogenous	Transient, non-significant increase in IL-10-producing cells.	(Thrower *et al.*, 2009)

			insulin use.		
GAD-alum	ROT1D	As above.	Higher C-peptide levels but no decrease in exogenous insulin use.	Increase in T_H2 cytokine profile. Increase in FOXP3 expression in PBMC	(Ludvigsson *et al.*, 2008) Follow-up by: (Axelsson *et al.*, 2010)
GAD-alum	ROT1D	As above.	None.	None.	(Wherrett *et al.*, 2011)
GAD-alum	ROT1D	As above.	None.	None.	(Ludvigsson *et al.*, 2012)

Table 8.1 Summary of clinical trials examining antigen-specific immunotherapies

However, as shown in Table 8.1, many studies have been conducted in ROT1D and one in at-risk relatives without any evidence of accelerated beta-cell autoimmunity.

8.3 Final conclusions

Although the exact cause for the defect in Treg function in T1D remains to be determined, there is much evidence to suggest it results from genetic predisposition, including the work in this thesis (although it may be exacerbated in disease states). As T1D is a heterogeneous and polymorphic disease it could be envisioned that future therapies will be personalised, for example, the adoptive transfer of *ex vivo* Tregs could be tailor-made according to the patients' genotype or even immunophenotype.

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